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14. ABSTRACT : There are contradictory observations on radiosensitivity in cancer stem cells vs. non-stem cells. To resolve these contradictory observations, we employed breast cancer stem cell (CSC)-like MDA-MB231 and MDA-MB453 cells as well as their corresponding non-stem cells. CSC-like cells proliferate without differentiating and have characteristics of tumor-initiating cells. These cells were exposed to γ-rays (1.25-8.75Gy) and survival curves were determined by colony formation. A final slope, D_0, of the survival curve for each cell line was determined to measure radiosensitivity. The D_0 of CSC-like and non-MDA-MB453 cells were 1.16 Gy and 1.55 Gy, respectively. Similar results were observed in MDA-MB231 cells. After determination of radiosensitivity, we investigated intrinsic cellular determinants which influence radiosensitivity. We further examined whether CSC-like cells are relatively radiosensitive owing to different intrinsic factors including cell cycle distribution, free-radical scavengers and DNA repair. We observed that even though cell cycle status and antioxidant content may contribute to differential radiosensitivity, differential DNA repair capacity may be a greater determinant. Unlike non-CSC cells, CSC-like cells have little/no sublethal damage repair and a low intracellular level of ATM. These results suggest that low DNA repair capacity is responsible for the high radiosensitivity of these CSC-like cells.

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Table of Contents

Introduction

Ionizing radiation and chemotherapeutic agents continue to be a frontline therapy for local control of breast cancer where surgery is either not possible or undesirable such as in breast conservation therapy. Previous studies suggest that the failure of conventional therapy is due to cancer stem (tumor-initiating) cells which are inherently resistant to radiation and chemotherapeutic agents (1-4). Bao et al. (1) reported that their radioresistance is mediated through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity. However, Ropolo et al. (5) claimed that cell cycle distribution and intracellular level of activated checkpoint proteins rather than DNA repair capacity contribute to the intrinsic radioresistant property of cancer stem cells (CSCs). Nevertheless, recent studies reveal that CSC may be more sensitive to radiation, rather than radioresistant, compared with established cancer cell lines (6-8). These discrepancies are probably due to dynamic properties of CSCs as well as limitations of experimental analytical techniques. During the granting period (3/1/2011-2/28/2013), we studied radiosensitivity in cancer stem cells and non-stem cells.

Body

We previously proposed to study determining radiosensitivity in cancer stem cells and non-stem cells on radiosensitivity *in vitro* and *in vivo*.

### *Characterization of CSC-like cells and non-stem cells*

Blocked CSC-like cells can proliferate without differentiating and have characteristics of tumor-initiating cells (1). This property arises as the result of stable transfection of the cells with a human Oct3/4 promoter driving the expression of GFP, although the mechanism of the block remains to be determined. In order to control for GFP expression, the corresponding non-CSC population was stably transfected with a plasmid expressing GFP under the control of a CMV immediate-early promoter (9). Both CSC-like and non-CSC populations could be readily shown to express high levels of GFP whereas the untransfected population did not (Fig. 1A). Figure 1B shows that CSC-like cells were also highly enriched CD44$^+$ and CD24$^-$ as previously described (9). In addition, as shown in Figure 1C, both sets of CSC-like cells also selectively expressed octamer binding transcription factor 3/4 (Oct-4), which is known to maintain CSC-like properties (10).

Figure 1. Characterization of breast cancer stem cell-like (CSC-like) cells. (**A**) Parental MDA-MB-453 cells were transfected with a plasmid encoding GFP under the control of the CMV promoter (non-stem) or Oct3/4 promoter (stem-like). After selection in G-418, GFP+ colonies were pooled. Phase-contrast images and fluorescence images of parental cells (Parent: a, d), CMV-GFP-transfected non-stem cells (Non-stem: b, e) or Oct3/4-GFP-transfected CSC-like cells (Stem-like: c, f) were visualized by light (**Phase-contrast: a-c**) or UV (**Fluorescence: d-f**) microscopy. (**B**) Flow cytometry characterization of parental, non-stem, or CSC-like cells was performed. CMV promoter-driven GFP cDNA (**e;** non-stem cells) or human Oct3/4 promoter-driven GFP cDNA (**f;** stem-like cells) transfected MDA-MB-453 cells were stained with surface marker antibodies (CD24, CD44) and evaluated by flow cytometry. (**a-c**) Unstained cells and (**a, d**) parental cells. (**C**) Stem cell-associated Oct-4 gene expression was examined in MDA-MB-453 and MDA-MB-231 parental (P), non-stem (N) and CSC-cell like (S) cells. Cells were harvested with lysis buffer. Lysates containing equal amounts of protein (20 µg/ml) were separated by SDS-PAGE, and immunoblotted with anti-Oct-4 antibody. Actin was shown as an internal standard.

Comparison of radiosensitivities of CSC-like cells and non-stem cells

To determine the radiosensitivity of MDA-MB-453 and MDA-MB-231 CSC-like and non-CSC cells, we used colony formation assay following exposure to γ-rays (Fig. 2A) and survival curves were plotted (Figs. 2B and C). A final slope, D_0, of the survival curve for each cell line was determined to measure radiosensitivity. D_0 of CSC-like MDA-MB-453 cells and that of non-CSC MDA-MB-453 cells were 1.16 Gy and 1.55 Gy, respectively (Fig. 2B). Similar results were observed in MDA-MB-231 cells (Fig. 2C). Our data clearly reveal that CSC-like cells are more sensitive to ionizing radiation than non-stem cells.


Figure 2. Survival curves for non-stem and CSC-like MDA-MB-453 and MDA-MB-231 cells after irradiation. (**A**) Colonies were obtained with non-stem and stem-like MDA-MB-453 cells. Cells were unirradiated (0 Gy) or irradiated (6.25 Gy), trypsinized, counted and plated. Cells were grown for 1-3 weeks and stained with crystal violet. (**B, C**) Survival curves for non-stem and stem-like MDA-MB-453 (**B**) and MDA-MB-231 (**C**) cells were determined after irradiation. Cells were exposed to various doses (1.25 Gy-8.75 Gy) of γ-radiation. Cells were trypsinized, counted and plated. Colony formation was determined 1-3 weeks after irradiation. Error bars represent standard error from the mean for three separate experiments.

Role of cell cycle distribution in differential radiosensitivity of CSC-like cells and non-stem cells

It has long been recognized that the degree of radiosensitivity is related to extrinsic factors (e.g., hypoxia) and intrinsic factors (e.g., cell cycle distribution, antioxidant levels, DNA repair capacity). We examined the role of these properties in the differential radiosensitivity of CSC-like cells and non-stem cells. Early studies in radiobiology had revealed that cells are most radiosensitive during M and G_2 phases and most resistant in late S phase (11). We investigated whether cell cycle distribution plays a role in radiosensitivity. Cell cycle distribution was measured by measuring DNA content after staining with propidium iodide (PI). Figure 3A shows that S population was 36.2% and 21.5% in non-stem and CSC-like cells, respectively, in MDA-MB-453 cells. Figure 3B shows that, in MDA-MB-231 cells, S population was 27.3% and 22.8% in non-stem and CSC-like cells, respectively. These results suggest that radioresistance in non-stem cells in both cell lines is associated with larger S population. This possibility was examined with synchronized cells. Treatment with 5 µM aphidicolin for 16 hr led to cell cycle arrest at the G_1 phase (Fig. 3C). Asynchronized and synchronized cells were irradiated at 6.25 Gy and survival was determined (Fig. 3D). Figure 3D shows that synchronized CSC-like cells were more sensitive to radiation than synchronized non-stem cells.
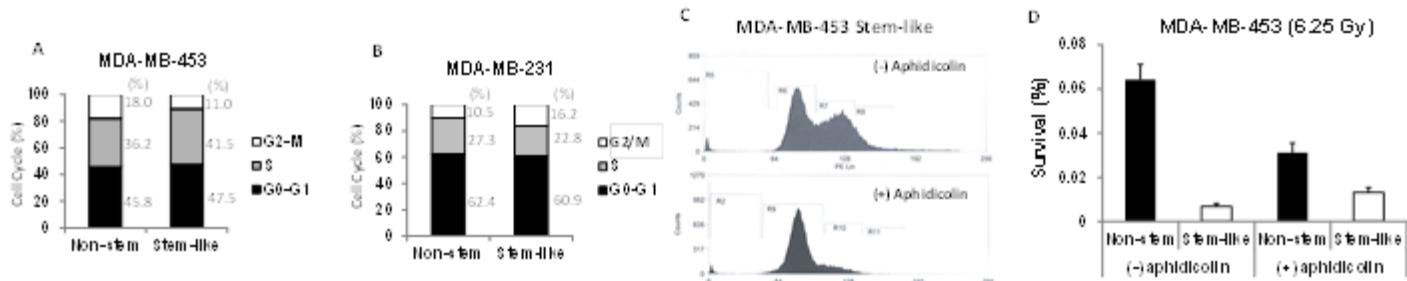
Figure 3. Flow cytometry analysis of cell cycle in asynchronous and aphidicolin-induced G_1 cell cycle arrest and radiosensitivity in non-stem and CSC-like cells. Flow cytometry was performed on non-stem and stem-like MDA-MB-453 cells (**A**) or MDA-MB-231 cells (**B**). The percentage of cells in the G_2/M, S, and G_1 were analyzed and plotted. Non-stem and CSC-like MDA-MB-453 cells were treated with aphidicolin (5 µM) for 16 hr. After aphidicolin treatment, cell cycle was analyzed (**C**) and radiation sensitivity was determined by

colony formation after irradiation (6.25 Gy) (**D**). Error bars represent standard error from the mean for three separate experiments.

Role of antioxidants in differential radiosensitivity of CSC-like cells and non-stem cells
Reactive oxygen species (ROS) are known to mediate the effect of ionizing radiation (12). ROS are normally controlled by the antioxidant defense system including the tripeptide glutathione and antioxidant enzymes such as catalase, MnSOD (manganese-containing superoxide dismutase) and CuZnSOD (copper-zinc-containing superoxide dismutase). We examined whether antioxidant status is related to differential radiosensitivity of CSC-like cells and non-stem cells. We observed that the levels of antioxidant enzymes in non-stem cells and CSC-like cells were equivalent (Figs. 4A and 4B). These results suggest that the levels of antioxidant enzymes are an unlikely determinant of differential radiosensitivity. Next, we investigated the role of glutathione content, in particular the reduced form (GSH). We observed that only approximately 1% of the total glutathione exists in oxidized form (GSSG) (data not shown). Figure 4C shows that unlike antioxidant enzymes, the intracellular level of GSH in non-stem cells was 1.29-fold higher than that in CSC-like cells. To examine whether GSH plays an important role in differential radiosensitivity of CSC-like cells and non-stem cells, both cells were treated with 200 µM L-buthionine-sulfoximine (BSO) for 24 hr and GSH content was determined. BSO, an inhibitor of GSH synthase, reduced the intracellular level of GSH by 89% and 94% in non-stem cells and CSC-like cells, respectively (Fig. 4C). The level of GSSG was almost undetectable in BSO-treated cells (data not shown). BSO-treated and untreated control cells were irradiated at 6.25 Gy and survival was determined as shown in Fig. 4D. BSO treatment sensitized cells to radiation in non-stem cells as well as CSC-like cells. However, although BSO reduced GSH content by 89% in non-stem cells, survival of BSO-treated non-stem cells was similar or higher than that of untreated CSC-like cells at 6.25 Gy irradiation. These results suggest that GSH content plays an important role in radiosensitivity. However, GSH content may not be a requisite factor in differential radiosensitivity of CSC-like cells and non-stem cells.


Figure 4. Role of anti-oxidant agents in radiosensitivity of non-stem cells and CSC-like cells. (**A**) Non-stem (N) and stem-like (S) MDA-MB-453 and MDA-MB-231 cells were harvested. Lysates containing equal amounts of protein (20 µg/ml) were separated by SDS-PAGE, and immunoblotted with anti-MnSOD, anti-CuZnSOD, or anti-catalase antibody. Actin was shown as an internal standard. (**B**) Densitometry analysis of each band was performed. The area integration of optical density of each band in stem-like cells (S) was compared with that in non-stem cells (NS). Error bars represent standard error from the mean for three separate experiments. (**C**) MDA-MB-453 non-stem cells and stem-like cells were treated with 200 µM L-buthionine-sulfoximine (BSO) for 24 hr and GSH content was determined. Error bars represent standard error from the mean for three separate experiments. (**D**) BSO-treated/untreated non-stem and stem-like MDA-MB-453 and MDA-MB-231 cells were irradiated at 6.25 Gy and survival was determined. Error bars represent standard error from the mean for three separate experiments.

Role of DNA repair capacity in differential radiosensitivity of CSC-like cells and non-stem cells
Previous studies have shown a good correlation between DNA repair capacity and radiosensitivity (13-15). We hypothesized that DNA repair capacity is a determining factor for differential radiosensitivity of CSC-like cells and non-stem cells. We investigated this possibility by examining sublethal damage repair. For this study, we chose single doses at 1% isosurvival: 6.25 Gy for CSC-like cells and 7.5 Gy for non-stem cells in MDA-MB-453 cells (Fig. 2B). To determine the capacity of DNA damage repair, the radiation dose was divided into two

fractions (3.75 Gy + 2.5 Gy for CSC-like cells and 5 Gy + 2.5 Gy for non-stem cells) separated by various time intervals (0.5-9 hr) at 24°C. Survival was determined after split-dose irradiation as shown in Fig. 5A. Figure 5A demonstrates that sublethal damage repair occurred in non-stem cells, but not in CSC-like cells. These data suggest an intrinsic difference between CSC-like cells and non-stem cells in terms of DNA repair capacity. This observation was confirmed in MDA-MB-231 CSC-like cells in which sublethal damage repair was also not observed after split-dose irradiation (Fig. 5B).

Figure 5. Analysis of sublethal damage repair. (**A**) Non-stem and stem-like MDA-MB-453 cells were exposed to two fractions of γ-radiation (5.0 + 2.5 Gy for non-stem and 3.75 + 2.5 Gy for stem-like) and incubated at 24°C for various time intervals between two exposures. Survival was compared to control group (irradiated without post-incubation) and plotted. Error bars represent standard error from the mean for three separate experiments. (**B**) Stem-like MDA-MB-453 and MDA-MB-231 were exposed to two fractions of γ-radiation (3.75 + 2.5 Gy) and incubated at 24°C for various time intervals between two exposures. Survival was compared to control group (irradiated without post-incubation) and plotted. Error bars represent standard error from the mean for three separate experiments.

Involvement of ATM in differential radiosensitivity

Previous studies have shown that ATM is responsible for sublethal damage repair (15, 16). To examine the involvement of ATM in differential radiosensitivity of CSC-like cells and non-stem cells, cells were irradiated at 8.75 Gy and phosphorylation (activation) of ATM was determined at various times (0.5-12 hr) thereafter. Data from immunoblot analysis shows that ATM was rapidly phosphorylated within 0.5 hr and then gradually dephosphorylated in CSC-like cells as well as non-stem cells (Figs. 6A and 6B). However, activating phosphorylation of ATM was significantly higher in non-stem cells than in CSC-like cells in both cell lines. Moreover, intracellular level of total ATM protein in non-stem cells was 5-6-fold higher than that in CSC-like cells, indicating that difference in intrinsic level of ATM might be responsible for differential radiosensitivity. This possibility was examined by treating cells with ATM inhibitor CP466722. MDA-MB-453 non-stem cells were pretreated with 100 μM CP466722 for 0.5 hr and then irradiated at 6.25 Gy. After irradiation, cells were incubated at 37°C for various times (0.5-12 hr) before western blot analysis (Fig. 6C). As shown in Figure 6C, ionizing radiation-induced phosphorylation of ATM was inhibited by 77% following treatment with CP466722. CP466722 treatment was not cytotoxic (data not shown), however, it reduced D_o from 1.5 Gy to 0.98 Gy (Fig. 6D). Similar results were observed in MDA-MB-231 non-stem cells (data not shown).


Figure 6. Ionizing radiation-induced phosphorylation of ATM and effect of ATM inhibitor CP466722 on radiosensitivity. (**A, B**) Non-stem and CSC-like MDA-MB-435 and MDA-MB-231 cells were irradiated at 8.75 Gy and phosphorylation (activation) of ATM was determined various times (0.5-12 hr) after irradiation.

Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-ATM or anti-phospho-ATM antibody. Actin was shown as an internal standard. (**C**) MDA-MB-453 non-stem cells were pretreated with/without 100 μM CP466722 for 30 min, irradiated at 6.25 Gy and incubated various times before immunoblot analysis as described above. (**D**) MDA-MB-453 non-stem cells were pretreated with/without 100 μM CP466722 for 30 min, irradiated at various doses (1.25 Gy-8.75 Gy) and incubated for 6 hr before colony formation analysis. Error bars represent standard error from the mean for three separate experiments.

## *Intracellular ATM and radiosensitivity*

We expanded our observations to determine if the differences in the intracellular level of ATM are due to decreased *ATM* gene expression or ATM protein stability. Data from semi-quantitative RT-PCR assay shows no significant differences in *ATM* gene expression (Figs. 7A and 7B). However, ATM protein stability was somewhat different. Figures 7C and 7D show that ATM protein in CSC-like cells degraded faster than that in non-stem cells. This is probably due to differences in ubiquitination activity. We further investigated the role of ATM in radiosensitivity by using the small hairpin RNA (shRNA) technique for ATM knockdown. MDA-MB-453 and MDA-MB-231 non-stem cells were infected with lentiviral vectors containing either control shRNA or ATM shRNAs. After puromycin-resistant cell clones were selected, ATM protein knockdown was verified by immunoblotting (upper panels of Fig. 8). Figure 8 shows that expression of ATM was not changed by control shRNA, but effectively reduced by ATM shRNA in both non-stem cells. We obtained several stable clones and chose control shRNA #2 and ATM shRNA #2 and #5 in MDA-MB-453 non-stem cells (Fig. 8A) and control shRNA #2 and ATM shRNA #1 and #4 in MDA-MB-231 non-stem cells (Fig. 8B). For radiosensitivity assay, cells were irradiated at 6.25 Gy and colony formation assay was performed. Figure 8 shows that there was no significant change in radiosensitivity in control shRNA clones compared with non-stem cells. In contrast, non-stem cells with ATM knockdown were significantly more sensitive to ionizing radiation than control non-stem cells. These data suggest that ATM plays an important role in the differential radiosensitivity of CSC-like cells and non-stem cells.

Figure 7. Determination of *ATM* gene expression and ATM protein stability in non-stem and CSC-like MDA-MB-435 and MDA-MB-231 cells. (**A, B**) Total RNA was extracted and reverse transcribed into cDNA. Human ATM mRNA was amplified and analyzed electrophoretically, and then quantified by Un-Scan-It gel software. (**C, D**) Cells were treated with 30 μg cycloheximide (CHM: >95% protein synthesis inhibition) for various times (2-16 hr) and harvested. Lysates containing equal amounts of protein (20 μg/ml) were separated

by SDS-PAGE, and immunoblotted with anti-ATM or anti-actin antibody. Actin was shown as an internal standard. Densitometry analysis of each band was performed as described in Figure 4.

A MDA-MB-453 Non-stem

B MDA-MB-231 Non-stem

Figure 8. Role of ATM in radiosensitivity in non-stem MDA-MB-453 (A) and MDA-MB-231 (B) cells. Non-stem cells were infected with control shRNA or ATM shRNA lentiviral particle (2.5×10^4-10^5 IFU) and stable clones were selected by treatment with 10-100 µg/ml puromycin. ATM knockdown level was assessed by immunoblot assay as described in Fig. 6 (upper panels) and survival was determined after irradiation at 6.25 Gy (lower panels). Error bars represent standard error from the mean for three separate experiments.

Quantitative analysis for ionizing radiation-induced DNA damage

It is well known that γ-phosphorylation of histone H2AX (γ-H2AX) "focus" formation is a rapid and sensitive cellular response to the presence of DNA double-strand breaks (DSBs) (17, 18). H2AX is one of the targets of ATM phosphorylation and γ-H2AX foci formation after ionizing radiation reflects DNA damage and repair (19). Figure 9 shows that γ-H2AX foci formation occurred rapidly within 0.5 hr after irradiation at 2.5 Gy and gradually reduced within 12 hr in both CSC-like and non-stem MDA-MB-453 cells. Nuclei containing at least six fluorescent foci were considered positive and kinetics of γ-H2AX foci removal after irradiation at 2.5 Gy or 8.75 Gy were analyzed in CSC-like cells and non-stem cells (Tables 1 and 2). As shown in Tables 1 and 2, the percentage of cells stained γ-H2AX reduced slowly in CSC-like cells in both cell lines. These results suggest that CSC-like cells have low DNA repair capacity which is responsible for the high radiosensitivity of these CSC-like cells. Similar results were observed with alkaline comet assay which detects DNA single- and double-strand breaks (Fig. 10A). After irradiation, 100-190 images were analyzed and % frequencies (linear) were plotted as a function of tail moments (logarithmic) (Fig. 10B). Dotted lines serve only to clarify the distributions, which is not to distinguish damaged or undamaged DNAs. When compared to non-stem cells (left column), stem-like cells (right column) show similar level of DNA damage (middle row), and the repair of DNA damage was blocked by ice (middle row) but progressed at 37°C (bottom row) (Fig. 10B). However, the efficiency of DNA repair appears to be much reduced for stem-like cells when compared to the repair of non-stem cells.

MDA-MB-453 (2.5 Gy)

Figure 9. Ionizing radiation-induced γ-H2AX foci formation. Non-stem and CSC-like MDA-MB-453 cells were irradiated at 2.5 Gy. After 0.5 hr or 12 hr incubation, phosphorylated H2AX was detected by immunofluorescent staining with anti-phospho-H2AX antibody. Nuclei were stained with DAPI.

6

Table 1

Percentage of cells stained γ-H2AX positive after irradiation at 2.5 Gy

MDA-MB-453	nonstem	stem-like
Control	10.0 ± 4.7	12.3 ± 0.6
0.5 hr	57.0 ± 4.5	85.9 ± 6.8
2 hr	30.0 ± 4.1	70.7 ± 9.4
6 hr	21.8 ± 8.3	41.3 ± 9.2
12 hr	10.4 ± 4.8	13.3 ± 9.1

MDA-MB-231	nonstem	stem-like
Control	12.7 ± 3.3	11.1 ± 4.0
0.5 hr	93.4 ± 2.0	94.2 ± 5.7
2 hr	57.9 ± 9.4	85.2 ± 5.9
6 hr	44.2 ± 8.0	67.8 ± 2.7
12 hr	26.0 ± 9.1	34.8 ± 6.9

Table 2

Percentage of cells stained γ-H2AX positive after irradiation at 8.75 Gy

MDA-MB-453	nonstem	stem-like
0 hr	10.7 ± 0.5	11.6 ± 1.0
0.5 hr	92.5 ± 7.6	96.6 ± 2.5
2 hr	96 ± 0.9	98 ± 1.3
6 hr	75 ± 3.4	88.4 ± 2.4
12 hr	54.8 ± 4.0	75.9 ± 5.0

MDA-MB-231	nonstem	stem-like
0 hr	10.9 ± 4.4	11.8 ± 4.8
0.5 hr	92.2 ± 0.7	92.9 ± 0.3
2 hr	96.2 ± 2.2	97.0 ± 1.3
6 hr	89.3 ± 4.2	92.5 ± 0.5
12 hr	76.6 ± 0.4	85.8 ± 1.3

Tables 1 and 2. Kinetics of γ-H2AX foci removal after irradiation. Non-stem and CSC-like MDA-MB-435 and MDA-MB-231 cells were irradiated at 2.5 Gy (**Table 1**) or 8.75 Gy (**Table 2**). Various times (0.5-12 hr) after irradiation, cells were fixed and immunostained with anti-phospho-H2AX antibody. Nuclei containing at least six fluorescent foci were considered positive and percentage of cells stained γ-H2AX positive was determined. Error bars represent standard error from the mean for three separate experiments.

Figure 10. Alkaline comet images and their quantitative analysis for non-stem and stem-like MDA-MB-231 cells after irradiation. (A) Control (0 Gy) or irradiated (6.25 Gy) cells for both non-stem cells (upper

row) and stem-like cells (bottom row) were subjected to alkaline comet assay (refer to the Methods) and the DNA was visualized by using a propidium iodide. (**B**) Distribution of comet tail moments for different treatments was plotted. Tail moments are the products of the distance of DNA migration (microns) and the amount of separated DNA (%).

Key research accomplishments

Our data demonstrate that (a) cell cycle status and antioxidant content may contribute to differential radiosensitivity , (b) unlike non-CSC cells, CSC-like cells have little/no sublethal damage repair and a low intracellular level of ATM, and (c) the intracellular level of ATM and DNA repair capacity are intrinsic cellular determinants which influence radiosensitivity in CSCs. These results suggest that heterogeneity of breast CSCs is due to an individual tumor's genetic makeup and is responsible for variation in intrinsic radiosensitivity of individual CSCs and outcome of radiotherapy.

Reportable outcomes

Manuscripts

1. Kim, S.Y., Rhee, J.G., Song, X., Prochownik, E.V., Spitz, D.R., and **Lee, Y.J.** Breast cancer stem cell-like cells are more sensitive to ionizing radiation than non-stem cells: role of ATM. PLoS One, 7, e50423, 2012. PMC3503893

2. Kim, S.Y., Kang, J.W., Song, X., Kim, B., Yoo, Y.D., Kwon, Y.T. and **Lee, Y.J.** Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. Cellular Signalling, 24, 961-969, 2013.

Presentations

1. Kim, S.Y., Pronchownik E.V. and **Lee, Y.J.** Permanently blocked cancer stem cell-like cells are sensitive to ionizing radiation. Presented at the 9th Annual Department of Surgery Research Day, Pittsburgh, PA, May 18, 2011.

2. Kim, S.Y., Pronchownik E.V., Spitz, D., and **Lee, Y.J.** Breast cancer stem cell-like cells are more sensitive to ionizing radiation than non-stem cells: role of ATM. Presented at the 103rd Annual Meeting of the American Association for Cancer Research in Chicago, IL, March 31-April 4, 2012.

3. Kim, S.Y., Kang, J.W., Song, X., Kim, B., Yoo, Y.D., Kwon, Y.T. and **Lee, Y.J.** Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. Presented at the 104th Annual Meeting of the American Association for Cancer Research in Washington DC, April 6-April 10, 2013.

Conclusion

In this study, we observed that CSC-like cells were more sensitive to ionizing radiation than their corresponding subset non-stem cells. Our data suggest that the lower levels of ATM in the CSCs likely explain their intrinsic radiosensitivity.

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Appendices

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Breast Cancer Stem Cell-Like Cells Are More Sensitive to Ionizing Radiation than Non-Stem Cells: Role of ATM

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Abstract

There are contradictory observations about the different radiosensitivities of cancer stem cells and cancer non-stem cells. To resolve these contradictory observations, we studied radiosensitivities by employing breast cancer stem cell (CSC)-like MDA-MB231 and MDA-MB453 cells as well as their corresponding non-stem cells. CSC-like cells proliferate without differentiating and have characteristics of tumor-initiating cells [1]. These cells were exposed to γ-rays (1.25–8.75 Gy) and survival curves were determined by colony formation. A final slope, D_0, of the survival curve for each cell line was determined to measure radiosensitivity. The D_0 of CSC-like and non-stem MDA-MB-453 cells were 1.16 Gy and 1.55 Gy, respectively. Similar results were observed in MDA-MB-231 cells (0.94 Gy vs. 1.56 Gy). After determination of radiosensitivity, we investigated intrinsic cellular determinants which influence radiosensitivity including cell cycle distribution, free-radical scavengers and DNA repair. We observed that even though cell cycle status and antioxidant content may contribute to differential radiosensitivity, differential DNA repair capacity may be a greater determinant of radiosensitivity. Unlike non-stem cells, CSC-like cells have little/no sublethal damage repair, a low intracellular level of ataxia telangiectasia mutated (ATM) and delay of γ-H2AX foci removal (DNA strand break repair). These results suggest that low DNA repair capacity is responsible for the high radiosensitivity of these CSC-like cells.

Introduction

Breast cancer is the most common cancer in American women, and the second leading cause of cancer death [2,3]. Due to improvement of early diagnosis with mammography and the development of more effective adjuvant therapies including radiation, the past 20 years have seen a significant decrease in mortality from breast cancer in the United States and elsewhere [3]. However, many women still suffer recurrence and incurable metastases, and the optimal management of these diseases remains undefined.

Ionizing radiation and chemotherapeutic agents continue to be a frontline therapy for local control of breast cancer where surgery is either not possible or undesirable such as in breast conservation therapy. Previous studies suggest that the failure of conventional therapy is due to cancer stem (tumor-initiating) cells which are inherently resistant to radiation and chemotherapeutic agents [4–7]. Bao et al. [4] reported that their radioresistance is mediated through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity. However, Ropolo et al. [8] claimed that cell cycle distribution and intracellular level of activated checkpoint proteins rather than DNA repair capacity contribute to the intrinsic radioresistant

property of cancer stem cells (CSCs). Nevertheless, recent studies reveal that CSC may be more sensitive to radiation, rather than radioresistant, compared with established cancer cell lines [9–11]. These discrepancies are probably due to dynamic properties of CSCs as well as limitations of experimental analytical techniques.

Breast CSCs have been well studied. The results of both Al-Hajj and colleagues and Ponti and colleagues suggest that breast cancer cells with the capacity for long-term self-renewal are enriched within the CD44+ (hyaluronan receptor), CD24− (P-selectin), and ESA+ (epithelial surface antigen) subset [12,13]. Because these breast CSCs are only a small portion (0.1–5%) of the population, it is extremely difficult to perform biochemical analysis and colony formation assay with CSCs. To resolve this difficulty, we employed permanently blocked cancer stem cells derived from two breast cancer cell lines. As previously described, CSC-like cells and their corresponding non-stem cells were generated by stable transfection of green fluorescent protein (GFP) under the control of the human octamer binding transcription factor 3/4 promoter (Oct3/4) and cytomegalovirus (CMV) promoter, respectively [1]. Interestingly, these CSC-like cells can proliferate without differentiation, have characteristics of tumor-initiating cells and express tumor cell markers (CD44+ and CD24−)

Figure 1. Characterization of breast cancer stem cell-like (CSC-like) cells. (A) Parental MDA-MB-453 cells were transfected with a plasmid encoding GFP under the control of the CMV promoter (non-stem) or Oct3/4 promoter (stem-like). After selection in G-418, GFP+ colonies were pooled. Phase-contrast images and fluorescence images of parental cells (Parent: a, d), CMV-GFP-transfected non-stem cells (Non-stem: b, e) or Oct3/4-GFP-transfected CSC-like cells (Stem-like: c, f) were visualized by light (**Phase-contrast: a–c**) or UV (**Fluorescence: d–f**) microscopy. **(B)** Flow cytometry characterization of parental, non-stem, or CSC-like cells was performed. CMV promoter-driven GFP cDNA (**e;** non-stem cells) or human Oct3/4 promoter-driven GFP cDNA (**f;** stem-like cells) transfected MDA-MB-453 cells were stained with surface marker antibodies (CD24, CD44) and evaluated by flow cytometry. (**a–c**) Unstained cells and (**a, d**) parental cells. **(C)** Stem cell-associated Oct-4 gene expression was examined in MDA-MB-453 and MDA-MB-231 parental (P), non-stem (N) and CSC-cell like (S) cells. Cells were harvested with lysis buffer. Lysates containing equal amounts of protein (20 µg/ml) were separated by SDS-PAGE, and immunoblotted with anti-Oct-4 antibody. Actin was shown as an internal standard. **(D)** Mammosphere formation was compared in MDA-MB-231 and MDA-MB-453 CSC-like and non-stem cells. For mammosphere formation, 1,000 cells from stem-like cells or non-stem cells were plated into ultra-low attachment plates. Phase-contrast images of mammospheres of non-stem (left panels) or CSC-like (right panels) cells were obtained 4 days or 9 days later. **(E)** Xenograft tumor formation was established with CSC-like and non-stem MDA-MB-231 cells. For tumor formation in NOD/SCID mice, 1×10^4 stem-like or non-stem cells were injected into the upper mammary fat pad. Tumor volumes were measured 30 days after injection.
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characteristic of CSCs [1]. These CSC-like cells and their isogenic non-CSC lines allow us to perform quantitative clonogenic survival assay and biochemical analysis.

In this study we observed that CSC-like cells were more sensitive to ionizing radiation than their corresponding subset non-stem cells. Our data suggest that the lower levels of ATM in the CSC-like cells likely explain their intrinsic radiosensitivity.

Materials and Methods

Cell Culture

Permanently blocked cancer stem cell (CSC)-like MDA-MB-453 and MDA-MB-231 cell lines were generated as previously described following stable transfection with a human Oct3/4

promoter driving the expression of green fluorescent protein (GFP) [1]. In brief, when cells were transfected with plasmids containing Oct3/4 promoter-driven GFP, G-418-resistant colonies were pooled and GFP-positive and GFP-negative cells were separated using a flow cytometer. GFP-positive cells were maintained in G418-containing DMEM or **RPMI**. GFP-positive cells were periodically subjected to flow cytometry to evaluate the fraction of GFP-positive cells. When cells were stably transfected with these plasmids, unexpectedly, these GFP-positive CSC-like cells were unable to differentiate and remained blocked in a CSC-like state. The mechanism still remains unknown of how permanently blocked CSC-like cells can be derived from breast cancer cell lines by expressing Oct3/4 promoter-driven GFP. As a control, the corresponding

Figure 2. Survival curves for non-stem and CSC-like MDA-MB-453 and MDA-MB-231 cells after irradiation. (A) Colonies were obtained with non-stem and stem-like MDA-MB-453 cells. Cells were unirradiated (0 Gy) or irradiated (6.25 Gy), trypsinized, counted and plated. Cells were grown for 1–3 weeks and stained with crystal violet. (B, C) Survival curves for non-stem and stem-like MDA-MB-453 (B) and MDA-MB-231 (C) cells were determined after irradiation. Cells were exposed to various doses (1.25 Gy-8.75 Gy) of γ-radiation. Cells were trypsinized, counted and plated. Colony formation was determined 1–3 weeks after irradiation. Error bars represent standard error from the mean for three separate experiments.
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non-CSC populations were generated by expressing GFP under the control of a CMV immediate-early promoter. The CSC-like cells can proliferate without differentiation and have characteristics of tumor-initiating cells. These cells were cultured in DMEM or RPMI 1640 with 10% FBS (HyClone, Logan, UT, USA) and 26 mM sodium bicarbonate for the monolayer cell culture. Petri-dishes containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2.

Mammosphere Formation

MDA-MB-231 or MDA-MB-453 CSC-like cells or non-stem cells were placed in ultra-low attachment 24 well culture plates (Corning, Lowell, MA, USA) for mammosphere formation assay.

Xenograft Tumor Formation

MDA-MB-231 CSC-like cells or non-stem cells (1×10^4 cells in 0.1 ml of sterile 0.9% NaCl and 0.1 ml of Matrigel) were injected into the right and left mammary fat pads of six-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Jackson Laboratories, Bar Harbor, ME, USA). All procedures involving the mice were in accordance with the Guide for the Care and Use of Laboratory Animals and on a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Reagents and Antibodies

L-Buthionine-sulfoximine (BSO), cycloheximide and aphidicolin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). CP466722 was purchased from Selleck Chemicals (Houston, TX, USA). Anti-ATM, anti-phosphorylated ATM and anti-phosphorylated H2AX antibody were from Cell Signaling (Beverly, MA, USA). Anti-manganese-containing superoxide dismutase (MnSOD) was purchased from Millipore (Billerica, MA, USA). Anti-copper-zinc-containing superoxide dismutase (CuZnSOD) was from Stressgen (Farmingdale, NY, USA). Anti-catalase was from Epitomics (Burlingame, CA, USA).

Fluorescence Microscopy

The morphological features and fluorescence signals for CSC-like and non-CSC cells were confirmed with phase contrast and fluorescence microscopy (Axiovert 40 CFL, Carl Zeiss Microimaging, NY, USA). The data were analyzed by microscope imaging processing software AxioVision from Zeiss.

CD44 and CD24 Staining

Immunostaining of MDA-MB-453 cell lines was performed with an APC-labeled monoclonal antibody (mAb) against CD44 and a PE-labeled mAb against CD24 (BD Biosciences, Inc., Franklin Lakes, NJ, USA). Staining was performed with recommended
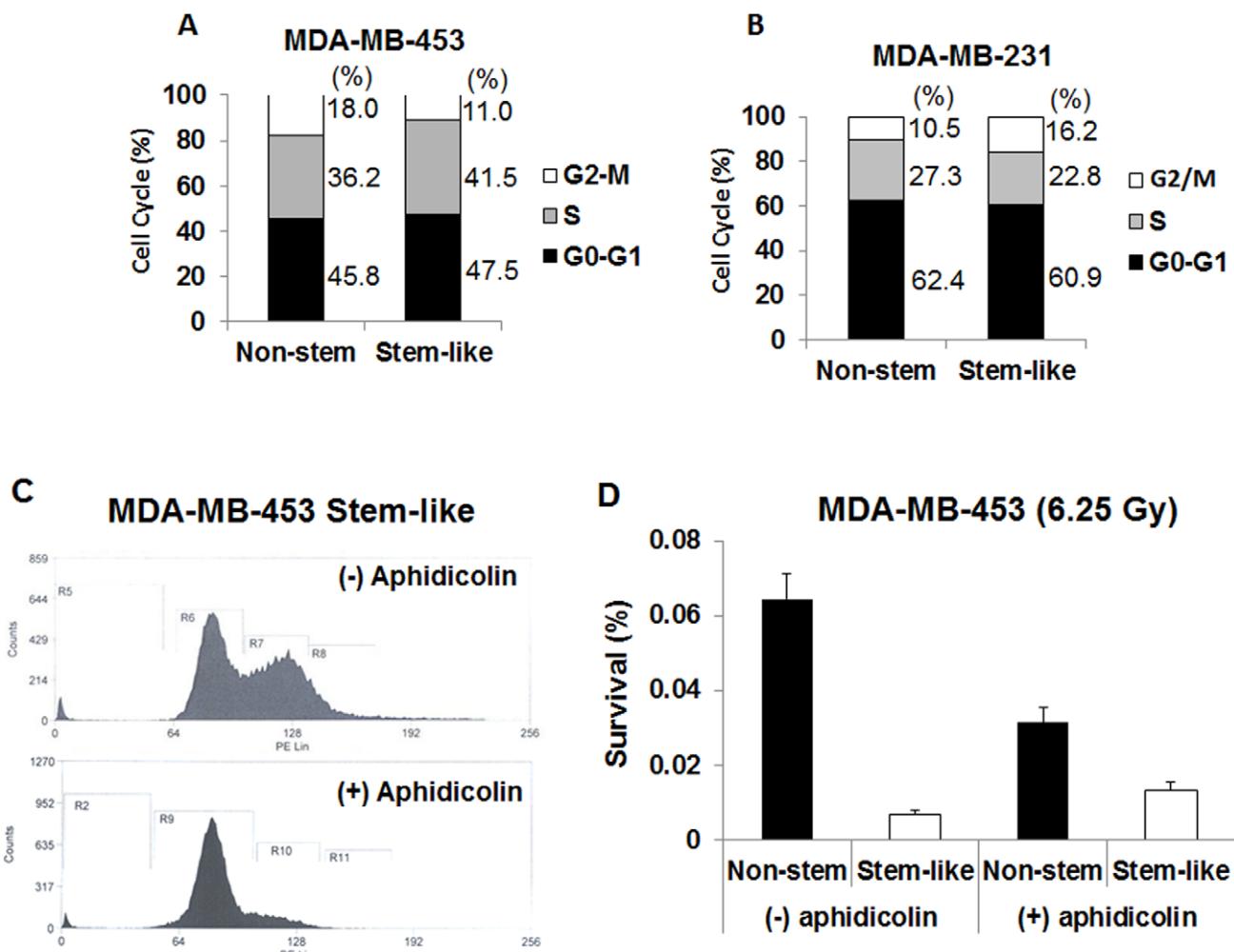
Figure 3. Flow cytometry analysis of cell cycle in asynchronous and effect of aphidicolin treatment on phase distribution and radiosensitivity in non-stem and CSC-like cells. Flow cytometry was performed on non-stem and stem-like MDA-MB-453 cells (**A**) or MDA-MB-231 cells (**B**). The percentages of cells in the G_2/M, S, and G_1 were analyzed and plotted. Non-stem and CSC-like MDA-MB-453 cells were treated with aphidicolin (5 µM) for 16 hr. After aphidicolin treatment, cell cycle was analyzed (**C**) and radiation sensitivity was determined by colony formation after irradiation (6.25 Gy) (**D**). Error bars represent standard error from the mean for three separate experiments.
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protocols of the supplier. Analysis was performed using the FACScan flow cytometer, and results were analyzed with CellQuest software (both from Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Protein Extracts and PAGE

Cells were scraped with 1×Laemmli lysis buffer (including 2.4 M glycerol, 0.14 M Tris (pH 6.8), 0.21 M SDS, and 0.3 mM bromophenol blue and boiled for 5 min. Protein concentrations were measured with BCA protein assay reagent (Pierce, Rockford, IL, USA). The samples were diluted with 1×lysis buffer containing 1.28 M β-mercaptoethanol, and an equal amount of protein was loaded on 8–12% SDS-polyacrylamide gels. SDS-PAGE analysis was performed according to Laemmli [14] using a Hoefer gel apparatus.

Immunoblot Analysis

Proteins were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes and blocked with 5% skim milk in TBS-Tween 20 (0.05%, v/v) for 30 minutes. The membrane was then incubated with antibodies against Oct 4, ATM, p-ATM (Cell Signaling, Danvers, MA, USA), or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1.5 hr. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the enhanced chemiluminescence protocol (ECL).

Densitometry Analysis

The Personal Densitometer SI from Molecular Dynamics was used to analyze the bands from immunoblotting assay. The ImageQuaNT program was used for the analysis.

Colony Formation Assay

For colony formation assay, CSC-like and non-CSC cells were exposed to ionizing radiation, trypsinized, counted, and plated at appropriate dilutions ($200–1\times10^6$ cells/dish). The dishes were incubated at 37°C for 7–21 days to allow colony formation. Colonies were fixed, stained and counted manually. For every surviving fraction, the plating efficiency (PE) value was normalized.

Figure 4. Role of anti-oxidant agents in radiosensitivity of non-stem cells and CSC-like cells. (**A**) Non-stem (N) and stem-like (S) MDA-MB-453 and MDA-MB-231 cells were harvested. Lysates containing equal amounts of protein (20 µg/ml) were separated by SDS-PAGE, and immunoblotted with anti-MnSOD, anti-CuZnSOD, or anti-catalase antibody. Actin was shown as an internal standard. (**B**) Densitometry analysis of each band was performed. The area integration of optical density of each band in stem-like cells (S) was compared with that in non-stem cells (NS). Error bars represent standard error from the mean for three separate experiments. (**C**) MDA-MB-453 non-stem cells and stem-like cells were treated with 200 µM L-buthionine-sulfoximine (BSO) for 24 hr and GSH content was determined. Error bars represent standard error from the mean for three separate experiments. (**D**) BSO-treated/untreated non-stem and stem-like MDA-MB-453 and MDA-MB-231 cells were irradiated at 6.25 Gy and survival was determined. Error bars represent standard error from the mean for three separate experiments.
doi:10.1371/journal.pone.0050423.g004

Figure 5. Analysis of sublethal damage repair. (**A**) Non-stem and stem-like MDA-MB-453 cells were exposed to two fractions of γ-radiation (5.0+2.5 Gy for non-stem and 3.75+2.5 Gy for stem-like) and incubated at 24°C for various time intervals between two exposures. Survival was compared to control group (irradiated without post-incubation) and plotted. Error bars represent standard error from the mean for three separate experiments. (**B**) Stem-like MDA-MB-453 and MDA-MB-231 were exposed to two fractions of γ-radiation (3.75+2.5 Gy) and incubated at 24°C for various time intervals between two exposures. Survival was compared to control group (irradiated without post-incubation) and plotted. Error bars represent standard error from the mean for three separate experiments.
doi:10.1371/journal.pone.0050423.g005

Figure 6. Ionizing radiation-induced phosphorylation of ATM and effect of ATM inhibitor CP466722 on radiosensitivity. (A, B) Non-stem and CSC-like MDA-MB-435 and MDA-MB-231 cells were irradiated at 8.75 Gy and phosphorylation (activation) of ATM was determined various times (0.5–12 hr) after irradiation. Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted with anti-ATM or anti-phospho-ATM antibody. Actin was shown as an internal standard. **(C)** MDA-MB-453 non-stem cells were pretreated with/without 100 μM CP466722 for 30 min, irradiated at 6.25 Gy and incubated various times before immunoblot analysis as described above. **(D)** MDA-MB-453 non-stem cells were pretreated with/without 100 μM CP466722 for 30 min, irradiated at various doses (1.25 Gy–8.75 Gy) and incubated for 6 hr before colony formation analysis. Error bars represent standard error from the mean for three separate experiments.
doi:10.1371/journal.pone.0050423.g006

Cell Cycle Phase Distribution Analysis

We performed the cell cycle analysis according to company recommendations. Briefly, cells were trypsinized and centrifuged at 1500 rpm for 5 min, washed twice with PBS and then fixed with 70% cold ethanol. Fixed cells were stained using PI/RNase Staining Buffer (BD Bioscience) and incubated for 15 min at room temperature before analysis. Analysis was performed using the FACScan flow cytometer, and results were analyzed with CellQuest software (both from Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Measurement of Glutathione

Cells were washed twice with ice cold PBS, scraped into cold PBS, and centrifuged at 4°C for 5 min at 400×g to obtain cell pellets, which were frozen at −80°C. Pellets were then thawed and homogenized in 50 mM potassium phosphate buffer, pH 7.8 containing 1.34 mM diethylenetriaminepentaacetic acid. Total glutathione content was determined by the method of Anderson [15]. Reduced and oxidized glutathione were distinguished by addition of 2 μl of a 1:1 mixture of 2-vinylpyridine and ethanol per 30 μl of sample followed by incubation for 1.5 hr and assay as previously described by Griffith [16]. All biochemical determinations were normalized to protein content using the method of Lowry *et al.* [17].

Irradiation

Cells were grown in 60-mm Petri dishes, which were placed on a turntable located 4.8 cm from a ^{137}Cs source in a vertical cylinder; dose rates during the period of these experiments were about 12 Gy/min.

Knock Down of ATM in Nonstem Cells with shRNA Lentiviral Infection

Three different ATM shRNA lentivirus vectors were obtained from Santa Cruz Biotechnology (cat. # sc-29761-V, Santa Cruz, CA, USA) along with the appropriate control vector (cat. # sc-108080). The infection procedure was performed according to the instructions provided by the company. After infection, stable clones were selected by treatment with puromycin. ATM knockdown level was assessed by western blot assay.

Immunostaining

After radiation, medium was discarded and cells were washed with 1×PBS buffer. Cells were fixed with 2% formaldehyde in PBS for 30 min at room temperature. Cells were washed again and added to 70% ethanol in PBS and then kept at −20°C in a freezer overnight. The next day, cells were blocked by using by 1% dry milk in PBS buffer containing Tween 20 and incubated for 30 min at room temperature. Cells were incubated with rabbit

Figure 7. Determination of *ATM* gene expression and ATM protein stability in non-stem and CSC-like MDA-MB-435 and MDA-MB-231 cells. (A, B) Total RNA was extracted and reverse transcribed into cDNA. Human ATM mRNA was amplified and analyzed electrophoretically, and then quantified by Un-Scan-It gel software. **(C, D)** Cells were treated with 30 µg cycloheximide (CHM: >95% protein synthesis inhibition) for various times (2–16 hr) and harvested. Lysates containing equal amounts of protein (20 µg/ml) were separated by SDS-PAGE, and immunoblotted with anti-ATM or anti-actin antibody. Actin was shown as an internal standard. Densitometry analysis of each band was performed as described in Figure 4.
doi:10.1371/journal.pone.0050423.g007

anti-γ-H2AX (Cell Signaling) primary antibody for 1 hr at room temperature. Following a wash in washing buffer (0.1% Tween 20 in PBS), the cells were incubated for 1 hr at room temperature in secondary antibody Alexa 555-conjugated goat anti-rabbit (Invitrogen, NY, USA) diluted in a 1:500 ratio. Cells were washed with washing buffer, stained with 0.5 µg/ml DAPI for 1 min for counter staining, and then mounted with cover glass. Immuno-fluorescent staining was observed and photographed using a FLUOVIEW FV1000 CONFOCAL MICROSCOPE (Filters-ALEXA 555 and DAPI) and software FV10-ASW version 02.01.01.04 interfaced to an Olympus (Olympus, Center Valley, PA, USA).

DNA Damage Assay

Ionizing radiation-induced DNA damage was assessed by the alkaline single-cell gel electrophoresis ("comet" assay) method. Cells were irradiated, trypsinized and embedded into 0.5% low-melting agarose on glass microscope slides. After treatment with alkaline lysis buffer, slides were subjected to electrophoresis, stained with propidium iodide (PI), and analyzed by epifluores-cence microscopy. For quantification of DNA damage, fluores-cence intensities (from PI staining) of the head and tail portions

were obtained from each comet image, and the percentage intensity of the tail portion was multiplied by the length of the tail (in µm) (DNA migration) to yield a tail moment.

Semi-quantitative Reverse Transcription-polymerase Chain Reaction Analysis

Total RNA was extracted and purified from cultured cells using the RNeasy Mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. The RNA was quantified by deter-mining absorbance at 260 nm. Two µg of total RNA from each sample was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit (Life Technologies, Inc.) in a volume of 20 µl. Human ATM mRNA was amplified using the sense primer 5′-CGT GCC AGA ATG TGA ACA CC-3′ and the antisense primer 5′-ACA GTA GCA GCC AAG GAC AC-3′. Human actin mRNA was amplified using the sense primer 5′-CTG GGA CGA CAT GGA GAA AA-3′ and the antisense primer 5′-AAG GAA GGC TGG AAG AGT GC-3′. The polymerase chain reaction (PCR) was carried out as follows: 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, followed by a 5-min extension stage at 72°C. Amplification products were
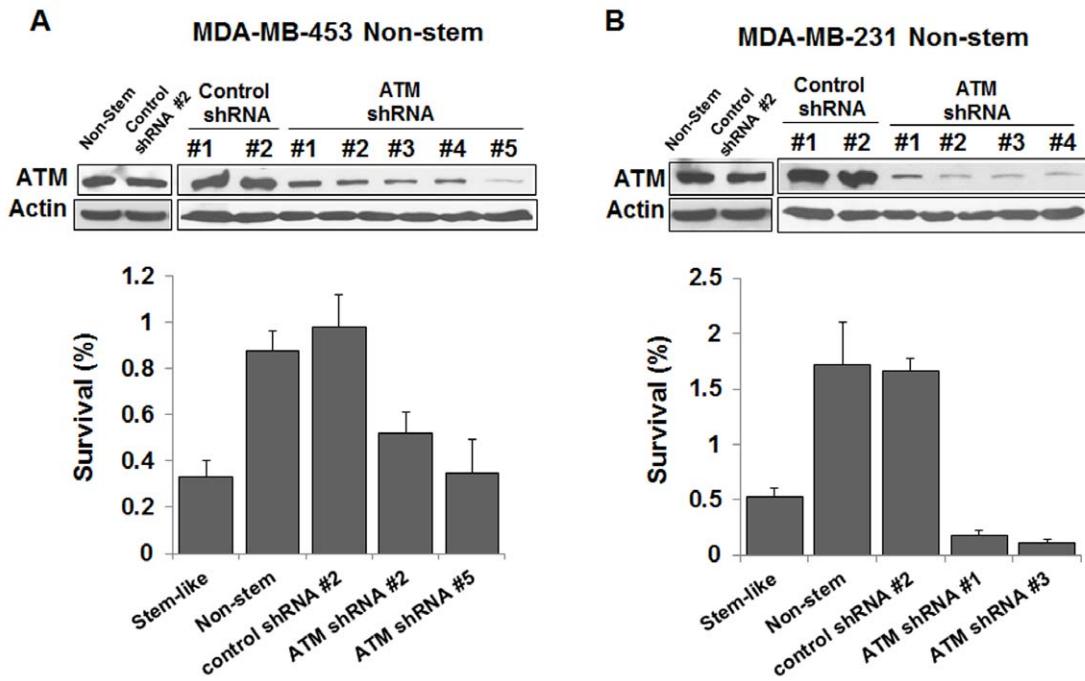
Figure 8. Role of ATM in radiosensitivity in non-stem MDA-MB-453 (A) and MDA-MB-231 (B) cells. Non-stem cells were infected with control shRNA or ATM shRNA lentiviral particle (2.5×10^4–10^5 IFU) and stable clones were selected by treatment with 10–100 µg/ml puromycin. ATM knockdown level was assessed by immunoblot assay as described in Fig. 6 (upper panels) and survival was determined after irradiation at 6.25 Gy (lower panels). Error bars represent standard error from the mean for three separate experiments.
doi:10.1371/journal.pone.0050423.g008

analyzed electrophoretically on 1.0% agarose gel containing 0.1 µg/ml ethidium bromide and then quantified by Un-Scan-It gel software (Silk Scientific Inc.).

Results

Characterization of CSC-like Cells and Non-stem Cells

Blocked CSC-like cells can proliferate without differentiating and have characteristics of tumor-initiating cells [1]. This property

Table 1. Percentage of cells stained γ-H2AX positive after irradiation at 2.5 Gy.

MDA-MB-453	Non-stem	Stem-like
0 hr	10.0±4.7	12.3±0.6
0.5 hr	57.0±4.5	85.9±6.8
2 hr	30.0±4.1	70.7±9.4
6 hr	21.8±8.3	41.3±9.2
12 hr	10.4±4.8	13.3±9.1
MDA-MB-231	**Non-stem**	**Stem-like**
0 hr	12.7±3.3	11.1±4.0
0.5 hr	93.4±2.0	94.2±5.7
2 hr	57.9±9.4	85.2±5.9
6 hr	44.2±8.0	67.8±2.7
12 hr	26.0±9.1	34.8±6.9

Kinetics of γ-H2AX foci removal after irradiation. Non-stem and CSC-like MDA-MB-435 and MDA-MB-231 cells were irradiated at 2.5 Gy. Various times (0.5–12 hr) after irradiation, cells were fixed and immunostained with anti-phospho-H2AX antibody. Nuclei containing at least six fluorescent foci were considered positive and percentage of cells stained γ-H2AX positive was determined. Error bars represent standard error from the mean for three separate experiments.
doi:10.1371/journal.pone.0050423.t001

Table 2. Percentage of cells stained γ-H2AX positive after irradiation at 8.75 Gy.

MDA-MB-453	Non-stem	Stem-like
0 hr	10.7±0.5	11.6±1.0
0.5 hr	92.5±7.6	96.6±2.5
2 hr	96±0.9	98±1.3
6 hr	75±3.4	88.4±2.4
12 hr	54.8±4.0	75.9±5.0
MDA-MB-231	**Non-stem**	**Stem-like**
0 hr	10.9±4.4	11.8±4.8
0.5 hr	92.2±0.7	92.9±0.3
2 hr	96.2±2.2	97.0±1.3
6 hr	89.3±4.2	92.5±0.5
12 hr	76.6±0.4	85.8±1.3

Kinetics of γ-H2AX foci removal after irradiation. Non-stem and CSC-like MDA-MB-435 and MDA-MB-231 cells were irradiated at 8.75 Gy. Various times (0.5–12 hr) after irradiation, cells were fixed and immunostained with anti-phospho-H2AX antibody. Nuclei containing at least six fluorescent foci were considered positive and percentage of cells stained γ-H2AX positive was determined. Error bars represent standard error from the mean for three separate experiments.
doi:10.1371/journal.pone.0050423.t002
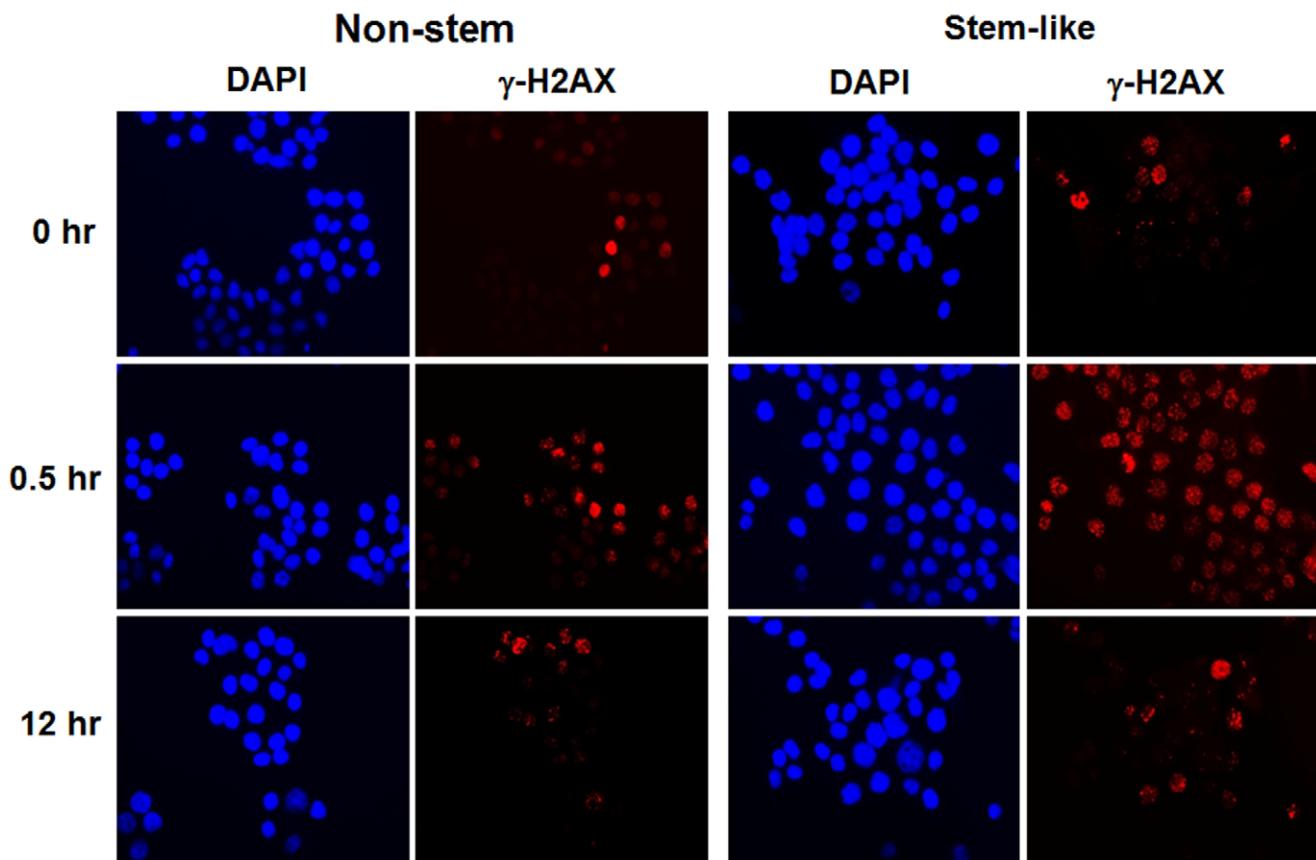
Figure 9. Ionizing radiation-induced γ-H2AX foci formation. Non-stem and CSC-like MDA-MB-453 cells were irradiated at 2.5 Gy. After 0.5 hr or 12 hr incubation, phosphorylated H2AX was detected by immunofluorescent staining with anti-phospho-H2AX antibody. Nuclei were stained with DAPI.
doi:10.1371/journal.pone.0050423.g009

arises as the result of stable transfection of the cells with a human Oct3/4 promoter driving the expression of GFP, although the mechanism of the block remains to be determined. In order to control for GFP expression, the corresponding non-CSC population was stably transfected with a plasmid expressing GFP under the control of a CMV immediate-early promoter [1]. Both CSC-like and non-CSC populations could be readily shown to express high levels of GFP whereas the untransfected population did not (Fig. 1A). Figure 1B shows that CSC-like cells were also highly enriched with CD44+ and CD24− as previously described [1]. In addition, as shown in Figure 1C, both sets of CSC-like cells also selectively expressed octamer binding transcription factor 3/4 (Oct-4), which is known to maintain CSC-like properties [18]. Dontu et al. [19] reported that nonadherent mammospheres are enriched in cells having functional characteristics of the self-renewal potential of stem cells. As shown in Figure 1D, CSC-like cells, but not non-stem cells, formed mammospheres very well. Mammospheres of CSC-like cells were grown faster than those of non-stem cells and the difference of mammosphere size between CSC-like cells and non-stem cells was about 45 times on day 9. Similar results were also observed during xenograft tumor formation and tumor growth. As shown in Figure 1E, in comparison with non-stem cells, CSC-like cells formed tumors earlier and xenograft tumors grew faster. The average tumor size from CSC-like cells was 4.6-fold larger than that from non-stem cells 30 days after transplantation into 10 NOD/SCID mice.

Comparison of Radiosensitivity of CSC-like Cells and Non-stem Cells

To determine the radiosensitivity of MDA-MB-453 and MDA-MB-231 CSC-like and non-CSC cells, we used colony formation assay following exposure to γ-rays (Fig. 2A) and survival curves were plotted (Figs. 2B and C). A final slope, D_0 (the dose required to reduce the number of clonogenic cells to 37% of their former value), of the survival curve for each cell line was determined to measure radiosensitivity. D_0 of CSC-like MDA-MB-453 cells and that of non-CSC MDA-MB-453 cells were 1.16 Gy and 1.55 Gy, respectively (Fig. 2B). As shown in Figure 2C, similar results were observed in MDA-MB-231 cells (0.94 Gy vs. 1.56 Gy). Our data clearly reveal that CSC-like cells are more sensitive to ionizing radiation than non-stem cells.

Role of Cell Cycle Distribution in Differential Radiosensitivity of CSC-like Cells and Non-stem Cells

It has long been recognized that the degree of radiosensitivity is related to extrinsic factors (e.g., hypoxia) and intrinsic factors (e.g., cell cycle distribution, antioxidant levels, DNA repair capacity). We examined the role of these intrinsic factors in the differential
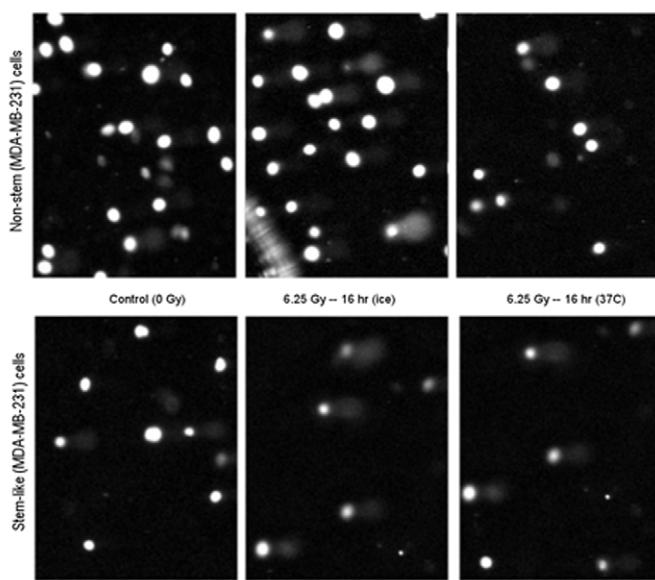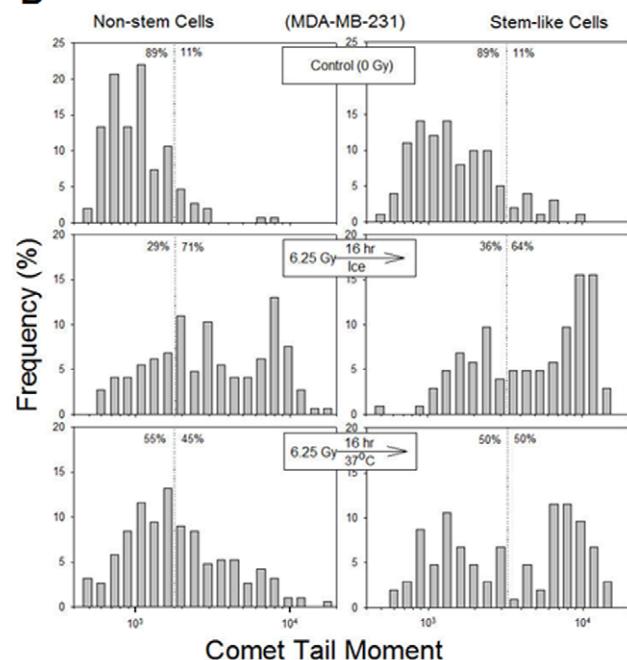
Figure 10. Alkaline comet images and their quantitative analysis for non-stem and stem-like MDA-MB-231 cells after irradiation. (A) Control (0 Gy) or irradiated (6.25 Gy) cells for both non-stem cells (upper row) and stem-like cells (bottom row) were subjected to alkaline comet assay (refer to the Methods) and the DNA was visualized by using a propidium iodide. **(B)** Distribution of comet tail moments for different treatments was plotted. Tail moments are the products of the distance of DNA migration (microns) and the amount of separated DNA (%). doi:10.1371/journal.pone.0050423.g010

radiosensitivity of CSC-like cells and non-stem cells. Early studies in radiobiology had revealed that cells are most radiosensitive during M and G_2 phases and most resistant in late S phase [20]. We investigated whether cell cycle distribution plays a role in radiosensitivity. Cell cycle distribution was measured by measuring DNA content after staining with propidium iodide (PI). Figure 3A shows that S population was 36.2% and 41.5% in non-stem and CSC-like cells, respectively, in MDA-MB-453 cells. Figure 3B shows that, in MDA-MB-231 cells, S population was 27.3% and 22.8% in non-stem and CSC-like cells, respectively. These results illustrate that even though S-phase may contribute somewhat in the determination of radiosensitivity, it may not be a major factor. We further examined whether cell cycle effects contribute to radiosensitivity with synchronized cells. Treatment with 5 µM aphidicolin for 16 hr, which didn't induce any significant cytotoxicity (data not shown), led to cell cycle arrest at the G_1 phase (Fig. 3C). Asynchronized and synchronized cells were irradiated at 6.25 Gy and survival was determined (Fig. 3D). Figure 3D shows that synchronized CSC-like cells were still more sensitive to radiation than synchronized non-stem cells.

Role of Antioxidants in Differential Radiosensitivity of CSC-like Cells and Non-stem Cells

Reactive oxygen species (ROS) are known to mediate the effect of ionizing radiation [21]. ROS are normally controlled by the antioxidant defense system including the tripeptide glutathione and antioxidant enzymes such as catalase, MnSOD (manganese-containing superoxide dismutase) and CuZnSOD (copper-zinc-containing superoxide dismutase). We examined whether antioxidant status is related to differential radiosensitivity of CSC-like cells and non-stem cells. We observed that the levels of antioxidant enzymes in non-stem cells and CSC-like cells were equivalent

(Figs. 4A and 4B). These results suggest that the levels of antioxidant enzymes are an unlikely determinant of differential radiosensitivity. Next, we investigated the role of glutathione content, in particular the reduced form (GSH). We observed that only approximately 1% of the total glutathione exists in oxidized form (GSSG) (data not shown). Figure 4C shows that unlike antioxidant enzymes, the intracellular level of GSH in non-stem cells was 1.29-fold higher than that in CSC-like cells. To examine whether GSH plays an important role in differential radiosensitivity of CSC-like cells and non-stem cells, both cells were treated with 200 µM L-buthionine-sulfoximine (BSO) for 24 hr and GSH content was determined. BSO, an inhibitor of GSH synthase, reduced the intracellular level of GSH by 89% and 94% in non-stem cells and CSC-like cells, respectively (Fig. 4C). The level of GSSG was almost undetectable in BSO-treated cells (data not shown). BSO-treated and untreated control cells were irradiated at 6.25 Gy and survival was determined as shown in Fig. 4D. BSO treatment sensitized cells to radiation in non-stem cells as well as CSC-like cells. However, although BSO reduced GSH content by 89% in non-stem cells, survival of BSO-treated non-stem cells was similar or higher than that of untreated CSC-like cells at 6.25 Gy irradiation. These results suggest that GSH content plays an important role in radiosensitivity. However, GSH content may not be a requisite factor in differential radiosensitivity of CSC-like cells and non-stem cells.

Role of DNA Repair Capacity in Differential Radiosensitivity of CSC-like Cells and Non-stem Cells

Previous studies have shown a good correlation between DNA repair capacity and radiosensitivity [3,22,23]. We hypothesized that DNA repair capacity is a determining factor for differential radiosensitivity of CSC-like cells and non-stem cells. We investi-

gated this possibility by examining sublethal damage repair. It is a well-documented observation that mammalian cells have the ability spontaneously to recover from sublethal low LET (linear energy transfer) ionizing radiation-induced damage [24]. A fractionation technique is usually used to test for sublethal damage repair [25,26]. For this study, we chose single doses at 1% isosurvival: 6.25 Gy for CSC-like cells and 7.5 Gy for non-stem cells in MDA-MB-453 cells (Fig. 2B). To determine the capacity of DNA damage repair, the radiation dose was divided into two fractions (3.75 Gy +2.5 Gy for CSC-like cells and 5 Gy +2.5 Gy for non-stem cells) separated by various time intervals (0.5–9 hr) at 24°C. Survival was determined after split-dose irradiation as shown in Fig. 5A. Figure 5A demonstrates that sublethal damage repair occurred in non-stem cells, but not in CSC-like cells. These data suggest an intrinsic difference between CSC-like cells and non-stem cells in terms of DNA repair capacity. This observation was confirmed in MDA-MB-231 CSC-like cells in which sublethal damage repair was also not observed after split-dose irradiation (Fig. 5B).

Previous studies have shown that ATM is responsible for sublethal damage repair [27,28]. To examine the involvement of ATM in differential radiosensitivity of CSC-like cells and non-stem cells, cells were irradiated at 8.75 Gy and phosphorylation (activation) of ATM was determined at various times (0.5–12 hr) thereafter. Data from immunoblot analysis shows that ATM was rapidly phosphorylated within 0.5 hr and then gradually dephosphorylated in CSC-like cells as well as non-stem cells (Figs. 6A and 6B). However, activating phosphorylation of ATM was significantly higher in non-stem cells than in CSC-like cells in both cell lines. Moreover, intracellular level of total ATM protein in non-stem cells was 5–6-fold higher than that in CSC-like cells, indicating that difference in intrinsic level of ATM might be responsible for differential radiosensitivity. This possibility was examined by treating cells with ATM inhibitor CP466722. MDA-MB-453 non-stem cells were pretreated with 100 μM CP466722 for 0.5 hr and then irradiated at 6.25 Gy. After irradiation, cells were incubated at 37°C for various times (0.5–12 hr) before western blot analysis (Fig. 6C). As shown in Figure 6C, ionizing radiation-induced phosphorylation of ATM was inhibited by 77% following treatment with CP466722. CP466722 treatment was not cytotoxic (data not shown), however, it reduced D_o from 1.5 Gy to 0.98 Gy (Fig. 6D). Similar results were observed in MDA-MB-231 non-stem cells (data not shown). We expanded our observations to determine if the differences in the intracellular level of ATM are due to decreased *ATM* gene expression or ATM protein stability. Data from semi-quantitative RT-PCR assay shows no significant differences in *ATM* gene expression (Figs. 7A and 7B). However, ATM protein stability was somewhat different. Figures 7C and 7D show that ATM protein in CSC-like cells degraded faster than that in non-stem cells. This is probably due to differences in ubiquitination activity. We further investigated the role of ATM in radiosensitivity by using the small hairpin RNA (shRNA) technique for ATM knockdown. MDA-MB-453 and MDA-MB-231 non-stem cells were infected with lentiviral vectors containing either control shRNA or ATM shRNAs. After puromycin-resistant cell clones were selected, ATM protein knockdown was verified by immunoblotting (upper panels of Fig. 8). Figure 8 shows that expression of ATM was not changed by control shRNA, but effectively reduced by ATM shRNA in both non-stem cells. We obtained several stable clones and chose control shRNA #2 and ATM shRNA #2 and #5 in MDA-MB-453 non-stem cells (Fig. 8A) and control shRNA #2 and ATM shRNA #1 and #4 in MDA-MB-231 non-stem cells (Fig. 8B). For radiosensitivity assay, cells were irradiated at 6.25 Gy and colony formation assay was

performed. Figure 8 shows that there was no significant change in radiosensitivity in control shRNA clones compared with non-stem cells. In contrast, non-stem cells with ATM knockdown were significantly more sensitive to ionizing radiation than control non-stem cells. These data suggest that ATM plays an important role in the differential radiosensitivity of CSC-like cells and non-stem cells.

It is well known that γ-phosphorylation of histone H2AX (γ-H2AX) "focus" formation is a rapid and sensitive cellular response to the presence of DNA double-strand breaks (DSBs) [29,30]. H2AX is one of the targets of ATM phosphorylation and γ-H2AX foci formation after ionizing radiation reflects DNA damage and repair [31]. Figure 9 shows that γ-H2AX foci formation occurred rapidly within 0.5 hr after irradiation at 2.5 Gy and gradually reduced within 12 hr in both CSC-like and non-stem MDA-MB-453 cells. Nuclei containing at least six fluorescent foci were considered positive and kinetics of γ-H2AX foci removal after irradiation at 2.5 Gy or 8.75 Gy were analyzed in CSC-like cells and non-stem cells (Tables 1 and 2). As shown in Tables 1 and 2, the percentage of cells stained γ-H2AX reduced slowly in CSC-like cells in both cell lines. These results suggest that CSC-like cells have low DNA repair capacity which is responsible for the high radiosensitivity of these CSC-like cells. Similar results were observed with alkaline comet assay which detects DNA single- and double-strand breaks (Fig. 10A). After irradiation, 100–190 images were analyzed and % frequencies (linear) were plotted as a function of tail moments (logarithmic) (Fig. 10B). Dotted lines serve only to clarify the distributions, which is not to distinguish damaged or undamaged DNAs. When compared to non-stem cells (left column), stem-like cells (right column) show similar level of DNA damage (middle row), and the repair of DNA damage was blocked by ice (middle row) but progressed at 37°C (bottom row) (Fig. 10B). However, the efficiency of DNA repair appears to be much reduced for stem-like cells when compared to the repair of non-stem cells.

Discussion

Several conclusions can be drawn upon consideration of the data presented here. First, CSC-like cells were more sensitive to ionizing radiation compared to their alternate subset non-stem cells. Second, although several factors have been known to determine cancer cell response to ionizing radiation, we observed that the main intrinsic determinant of differential radiosensitivity was DNA repair capacity, in particular ATM level, rather than cell cycle status or antioxidant levels.

Colony formation (clonogenic) assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. The assay essentially tests every cell in the population for its ability to undergo "unlimited" division. This assay is the method of choice to determine cell reproductive death after treatment with ionizing radiation. It is well known that colonies from irradiated cells vary in size (Fig. 2A). This is probably due to radiation-induced cell cycle arrest. Interestingly, post-irradiated colonies appear to stain less densely than pre-irradiated colonies in non-stem cells. It is possible that an increased ATM activity promotes changes associated with epithelial-mesenchymal transdifferentiation (EMT) and results in increased cell mobility [32].

Early studies in radiation biology employed both *in vitro* and *in vivo* models to reveal that various determinant factors contribute to differential radiosensitivity. These factors are both extrinsic and intrinsic and include the tumor microenvironment (e.g., hypoxia and interaction with stromal elements) and radiation response

elements (e.g., cell cycle distribution, antioxidant content, and DNA repair capacity) [3,7,33–35].

Iida et al. [36] revealed that hypoxia-induced cancer stem cell marker CD133 gene expression is mediated through OCT- and SRY(sex-determining region Y)-binding sites on P1 promoter. Oct-4 and SRY-box containing gene 2 (SOX-2) directly binds to OCT- and SRY binding sites, respectively, on P1 promoter and up-regulates hypoxia-induced promoter activity of CD133 gene expression. Our data in Figure 1C shows Oct-4 gene expression in both CSC-like cell lines. It is possible that Oct-4 may regulate cancer stem cell maker CD44 gene expression. This possibility needs to be further investigated.

The position of tumor cells within the cell cycle confers radiosensitivity. For instance, the late G_2 and M phases are generally thought to be the most radiosensitive and the late S phase the most radioresistant. Al-Assar et al. [35] reported that breast cancer stem-like cells have a larger S-G_2 fraction. Although cell cycle distribution may contribute somewhat to differential radiosensitivity, our data with synchronized cells suggest this to be a minor factor (Fig. 3D).

Antioxidants are well known to have a protective effect against radiation damage. MnSOD is considered to be one of the most important intracellular antioxidant enzymes and is localized to mitochondria. CuZnSOD is an intracellular enzyme mainly localized to cytosol. Catalase can decompose H_2O_2 which is made by living organisms exposed to oxygen, to water and oxygen. Data from Figure 4A shows no significant differences in the intracellular levels of antioxidant enzymes between CSC-like cells and non-stem cells. GSH is known to be the major ROS-scavenging system in cells. Nguyen et al. [37] observed an increased expression of genes involved in GSH synthesis in CSC suggesting that the intracellular level of GSH is responsible for radioresistance. Indeed, the lowering of endogenous GSH content by BSO treatment enhanced radiosensitivity in both CSC and

non-stem cells ([3]; Figs. 4C and 4D). Nonetheless, data from Figure 4D shows that BSO-treated non-stem cells were still more resistant to radiation than BSO-treated CSC-like cells.

Bao et al. [3] demonstrated that radioresistance of glioma stem cells is mediated through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity. Also, Ropolo et al. [8] observed that glioma stem cells display an elongated cell cycle and enhanced basal activation of checkpoint proteins that might contribute to their radioresistance. These studies suggest that radiosensitivity features are probably dynamic in nature. In this study, we observed a differential level of ATM in CSC-like cells and non-stem cells in MDA-MB-453 and MDA-MB-231 cell lines. Our data suggest that the intracellular level of ATM and/or its phosphorylation-dependent (activation) act as determinants of radiosensitivity. It still remains unclear, however, why non-stem cells have higher levels of ATM compared with CSC-like cells. At the present time, we can only speculate on the differential ATM gene expression in CSC-like cells and non-stem cells. Previous studies demonstrated that CSC-associated expression of genes such as NOTCH and WNT is responsible for radioresistance [3,37–41]. In these CSC-like cells, CSC-associated gene expression may down-regulate ATM gene expression.

The studies presented here further elucidate the ATM gene regulation mechanisms involved in radiosensitivity. The differential radiation response in CSC-like cells and non-stem cells provides a useful model system for further investigation of this issue.

Author Contributions

Conceived and designed the experiments: SYK YJL. Performed the experiments: SYK JGR XS. Analyzed the data: SYK JGR DRS YJL XS. Contributed reagents/materials/analysis tools: EVP DRS. Wrote the paper: SYK YJL.

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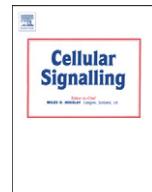
Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells

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ABSTRACT

Previous studies have demonstrated that a small subset of cancer cells is capable of tumor initiation. The existence of tumor initiating cancer stem cells (CSCs) has several implications in terms of future cancer treatment and therapies. However, recently, several researchers proposed that differentiated cancer cells (non-CSCs) can convert to stem-like cells to maintain equilibrium. These results imply that removing CSCs may prompt non-CSCs in the tumor to convert into stem cells to maintain the equilibrium. Interleukin-6 (IL-6) has been found to play an important role in the inducible formation of CSCs and their dynamic equilibrium with non-stem cells. In this study, we used CSC-like human breast cancer cells and their alternate subset non-CSCs to investigate how IL-6 regulates the conversion of non-CSCs to CSCs. MDA-MB-231 and MDA-MB-453 CSC-like cells formed mammospheres well, whereas most of non-stem cells died by anoikis and only part of the remaining non-stem cells produced viable mammospheres. Similar results were observed in xenograft tumor formation. Data from cytokine array assay show that IL-6 was secreted from non-CSCs when cells were cultured in ultra-low attachment plates. IL-6 regulates CSC-associated OCT-4 gene expression through the IL-6-JAK1-STAT3 signal transduction pathway in non-CSCs. Inhibiting this pathway by treatment with anti-IL-6 antibody (1 μg/ml) or niclosamide (0.5–2 μM)/LLL12 (5–10 μM) effectively prevented OCT-4 gene expression. These results suggest that the IL-6-JAK1-STAT3 signal transduction pathway plays an important role in the conversion of non-CSCs into CSCs through regulation of OCT-4 gene expression.

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1. Introduction

Breast cancer has become the most frequently diagnosed cancer among women in the United States [1,2]. The past decades have seen advances in the diagnosis and therapeutic treatment of breast cancer. Despite this progress, breast cancer is still a leading cause of cancer-related deaths among women, with as many 40% relapsing

with cancer recurrence and subsequent metastatic disease [2,3]. The major cause of death is not from the primary tumor itself but from the cancer cells at the distant sites to which the cancer has migrated [4]. Recent research in the past few years shows that breast cancer stem cells may be the cause of the recurrence and metastasis, which results in the spread of cancer to other parts of the body such as the bones, liver, brain, and lungs [5–7]. The concept of cancer stem cells being responsible for tumor origin, maintenance, and resistance to treatment has gained prominence in the field of cancer research [3,8–10]. These cancer stem cells represent a minor subpopulation of cells in the tumor and are also distinct from the more differentiated tumor cells. Traditional cancer treatments are effective at reducing tumor mass but often fail to produce long-term clinical complete remissions, possibly due to their inability to eliminate the cancer stem cell population [3]. Therefore, targeted therapy for cancer stem cells has been proposed to improve the efficacy of cancer treatments [11–13]. The therapeutic targeting of cancer stem cells may have the potential to remove residual disease and become an important component of a multimodality treatment.

For a long time, human cancers have been recognized as a morphologically heterogeneous population of cells [14]. At least two models of tumor growth have been used to explain the heterogeneous potential of tumor cells and the process of metastasis in general [15–18]. The stochastic model claims that tumors are originally biologically homogeneous. However, according to random or stochastic influences that alter the behavior of individual cells in tumor, tumors can gain functional heterogeneity. These influences can be intrinsic (levels of transcription factors and signaling pathways) or extrinsic (microenvironment and immune response) [19–21]. The hierarchical model assumes that only a very small subpopulation such as cancer stem cells within the tumor actually has the capacity to initiate and sustain tumor growth. In contrast, the bulk heterogeneous tumor population is not tumorigenic and only cancer stem cells are able to grow primary tumor and metastatic tumor [22–24]. Interestingly, several researchers proposed that differentiated cancer cells can convert to stem-like cells to maintain equilibrium [25,26]. Iliopoulos et al. [26] reported the inducible formation of breast CSCs and their dynamic equilibrium with non-CSCs via IL-6 secretion.

In this study, we observed that breast CSC-like cells were the major contributors to tumorigenicity. However, our data showed that even non-CSCs can contribute to tumor formation. Our mammosphere formation data showed that secreted IL-6 from non-CSCs activates the JAK1-STAT signal transduction pathway and upregulates CSC-associated OCT-4 gene expression. These results support the proposal that part of the non-CSC population can convert to CSC-like cells to maintain an equilibrium state and subsequently these CSC-like cells can initiate tumor formation.

2. Materials and methods

2.1. Cell culture

Permanently blocked cancer stem cell (CSC)-like MDA-MB-231 human breast adenocarcinoma and MDA-MB-453 human breast carcinoma cell lines, which can proliferate without differentiation and have characteristics of tumor-initiating cells, were generated in Dr. Prochownik's laboratory as previously described following stable transfection with a human Oct3/4 promoter driving the expression of green fluorescent protein (GFP) [27] and their corresponding non-CSC cell lines were generated by stable transfection of DsRed-Monomer N1 (cat. 632465, Clontech, CA, USA) using Lipofectamine 2000 reagent (Invitrogen, NY, USA). Stably transfected clones were selected, examined for expression of tumor markers (CD44, CD24, and Oct-4), pooled, and maintained with G418 (800–1000 μg/ml, Cellgro, VA, USA). The cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 or Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 26 mM sodium bicarbonate for the monolayer cell culture. Petri dishes containing cells were kept in a 37 °C humidified incubator with a mixture of 95% air and 5% CO_2.

2.2. Drug treatment

Niclosamide (5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide) and LLL12 (5-hydroxy-9,10-dioxo-9,10-dihydroanthracene-1-sulfonamide) were purchased from Biovision (Milpitas, CA). These drugs were dissolved in dimethylsulfoxide (DMSO) and applied to cells. Treatment of cells with drugs was accomplished by aspirating the medium and replacing it with medium containing these drugs.

2.3. Fluorescence microscopy

The morphological features and fluorescence signals for CSC-like and non-CSC cells were confirmed with phase contrast and fluorescence microscopy (Axiovert 40 CFL, Carl Zeiss Microimaging, NY, USA).

The data were analyzed by microscope imaging processing software AxioVision from Zeiss.

2.4. Protein extracts and PAGE

Cells were scraped with $1\times$ Laemmli lysis buffer (including 2.4 M glycerol, 0.14 M Tris (pH 6.8), 0.21 M SDS, and 0.3 mM bromophenol blue) and boiled for 5 min. Protein concentrations were measured with BCA protein assay reagent (Pierce, Rockford, IL, USA). The samples were diluted with $1\times$ lysis buffer containing 1.28 M β-mercaptoethanol, and an equal amount of protein was loaded on 8–12% SDS-polyacrylamide gels. SDS-PAGE analysis was performed using a Hoefer gel apparatus.

2.5. Immunoblot analysis

Proteins were separated by SDS-PAGE, electrophoretically transferred to mitrocellulose membranes and blocked with 5% skim milk in TBS-Tween 20 (0.05%, v/v) for 30 min. The membrane was incubated with antibodies against anti-JAK-1, anti-phospho-JAK1, anti-STAT3, anti-phospho-STAT3, anti-Oct 4 (Cell Signaling, Danvers, MA, USA), or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the enhanced chemiluminescence protocol.

2.6. Mammosphere formation assay

Prior to mammosphere formation, CSC-like and non-CSC breast cancer cells were grown as a monolayer culture as described above. One thousand cells were trypsinized and plated to an ultra-low attachment 24 well plate (Corning, Lowell, MA, USA). After 4–10 days, the mammosphere formation was observed. For comparison of the mammosphere size for stem-like and non-stem cancer cells, we used the Adobe photoshop program (Adobe Photoshop CS3, San Jose, CA, USA).

2.7. Aldefluor assay and flow cytometry

To measure and isolate cells with high aldehyde dehydrogenase (ALDH) activity, the Aldefluor assay was performed according to the manufacturer's guidelines (Stemcell Technologies, NC, USA). Dissociated single cells were suspended in Aldefluor assay buffer containing the ALDH substrate, bodipy aminoacetaldehyde (BAAA), at 1.5 mM and incubated for 40 min at 37 °C. To distinguish between ALDH-positive and ALDH-negative cells, a fraction of cells was incubated under identical condition in the presence of a 10-fold molar excess of the ALDH inhibitor, diethylaminobenzaldehyde (DEAB), which resulted in a significant decrease in the fluorescence intensity of ALDH-positive cells and was used to compensate the flow cytometry. Analysis was performed using the FACScan flow cytometer, and results were analyzed with CellQuest software (both from Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA).

2.8. Annexin V binding

Phosphatidylserine externalization, a marker of apoptotic events, was detected by binding of allophycocyanin (APC)-conjugated Annexin V. Non-stem cells were plated into ultra-low attachment plates, incubated various days, and stained with mouse anti-human annexin V antibody. Cells were analyzed by flow cytometry. Typically, 100,000 events were collected using excitation/emission wavelengths of 488/525 nm.

2.9. Immunofluorescent staining

Mammospheres of CSC-like cells and non-stem cells were harvested by cytospin and fixed in 2% paraformaldehyde for 15 min. These slides were washed 3 times in $1 \times$ PBS buffer, and then permeabilized in 0.5% Triton for 10 min. After further washing in PBS, using 1% BSA in PBS buffer, cells were blocked for 30 min. Cells were incubated with Oct-4 primary antibody (Abcam, MA, USA) during 1 h at room temperature. Following a wash in PBS, the cells were incubated for 1 h at room temperature in secondary antibody Alexa 647-conjugated goat anti-rabbit (Invitrogen, NY, USA) diluted 1:500 ratio in PBS. Cells were washed with PBS buffer, stained with 0.5 μg/ml DAPI (4′, 6-diamidino-2-phenylindole, Invitrogen) for 1 min, washed again 3 times and then covered with mounting medium (Vector Laboratories). Immunofluorescent staining was observed and photographed using an FluoView FV1000 confocal microscope (Filters-Alexa 647 and DAPI) and software FV10-ASW version 02.01.01.04 interfaced to an Olympus (Olympus, PA, USA).

2.10. Animal model

For xenograft tumor formation, MDA-MB-231 CSC-like cells or non-stem cancer cells (1×104 cells in 0.1 ml of sterile 0.9% NaCl and 0.1 ml of Matrigel) were injected into the upper mammary fat pads of six-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Jackson Laboratories, Bar Harbor, ME, USA). All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (Permit Number: 101904).

2.11. Cryosection and immunoblot analysis in tissue

Immediately after sacrifice the formed tumors were removed and cut in two burdens for the cryosection and western blot analysis. For the cryosection, one half was fixed in 2% paraformaldehyde overnight at 4 °C and then soaked in 30% sucrose solution for an additional 4 h at 4 °C. The frozen tumors were cut to 8 μm thickness by Microm cryostat (Microm International, Walldorf, Germany) and examined by fluorescence microscope. The other half tumor was homogenized and dissolved in SDS lysis buffer. Lysates containing equal amounts of protein from tumor tissues were separated by SDS-PAGE and immunoblotted with anti-Oct-4 antibody.
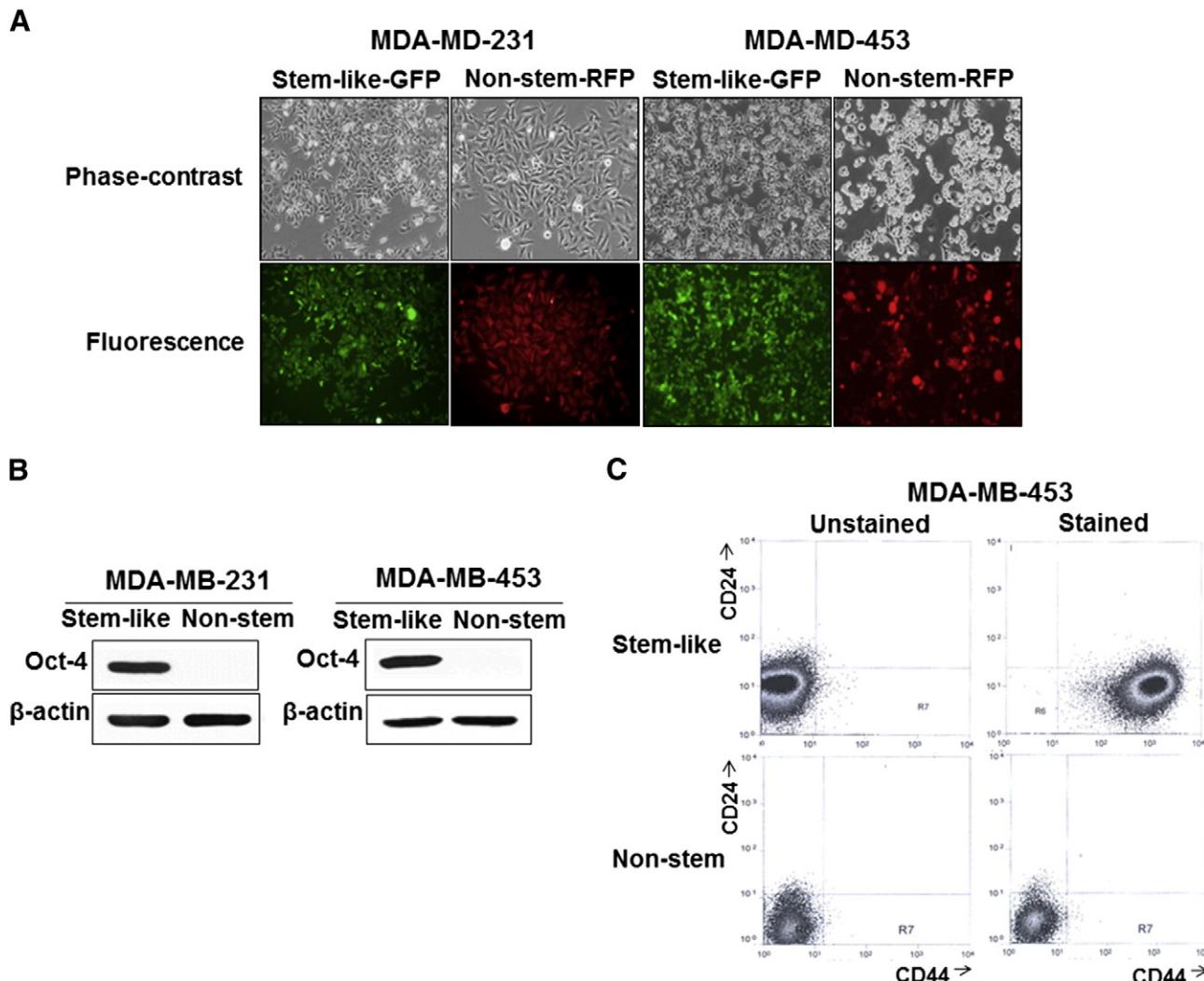
Fig. 1. Characterization of MDA-MB-231 and MDA-MB-453 cancer stem cell-like (CSC-like) and non-stem cancer (non-CSC) cells. (A) Cells were transfected with a plasmid encoding GFP under the Oct-3/4 promoter for selection of stem-like cells or RFP encoding under the control of the CMV promoter for selection of non-stem cells. After selection using G418, positive colonies were verified by tumor marker (CD44, CD24, and Oct-4) expression and then pooled. Phase-contrast images or fluorescence images of Oct-3/4-GFP-transfected stem-like cells (left panels) and CMV-RFP-transfected non-stem cells (right panels) were visualized by light (phase-contrast) or UV (fluorescence) microscopy, respectively. (B) Stem-associated Oct-4 gene expression was examined in stem-like cells (left side) and non-stem cells (right side) by western blot analysis. Actin was shown as an internal standard. (C) Flow cytometry characterization of CSC-like or non-stem cells was performed by staining with surface marker antibodies (CD24, CD44) and evaluated by flow cytometry.

2.12. Cytokine array assay

To detect the levels of cytokines and growth factors in MDA-MB-231 non-stem or stem-like cell-conditioned media, antibody-based cytokine array system (Raybio Human Cytokine Antibody Array 3, RayBiotech, Norcross, GA) was used following the manufacturer's instructions. Briefly, cells were cultured by RPMI media including 10% FBS in 6 well plate to 60–70% confluence. Fresh media was placed on the cells for 36 h and then collected. To remove any floating cells, collected media was centrifuged at 1000 g for 5 min. The supernatant was collected again, and used in the array experiment. The assay membranes were incubated in blocking solution for 30 min at room temperature. Each conditioned media sample was added to the membrane and incubated with gentle rocking at 4 °C overnight. After washing the membrane, biotin-conjugated anti-cytokine antibody was added to each membrane for 2 h at room temperature. Again washing the membrane, HRP-conjugated streptavidin

secondary antibody reaction was allowed to proceed for 2 h at room temperature by rocking. Cytokines bound to membranes were evaluated by chemiluminescence assay. Signal quantification was measured by subtracting the background signal using the UN-SCAN-IT program (Silk Scientific, Orem, Utah).

2.13. Neutralization with anti-IL-6 antibody

MDA-MB-231 non-CSCs were plated into ultra-low attachment plates and treated with anti-IL-6 antibody (Biolegend, San Diego, CA).

2.14. Statistical analysis

Statistical analysis was carried out using GraphPad InStat 3 software (GraphPad Software, Inc., San Diego, CA, USA). Significance was set at values of $p<.01$ or $p<.001$.
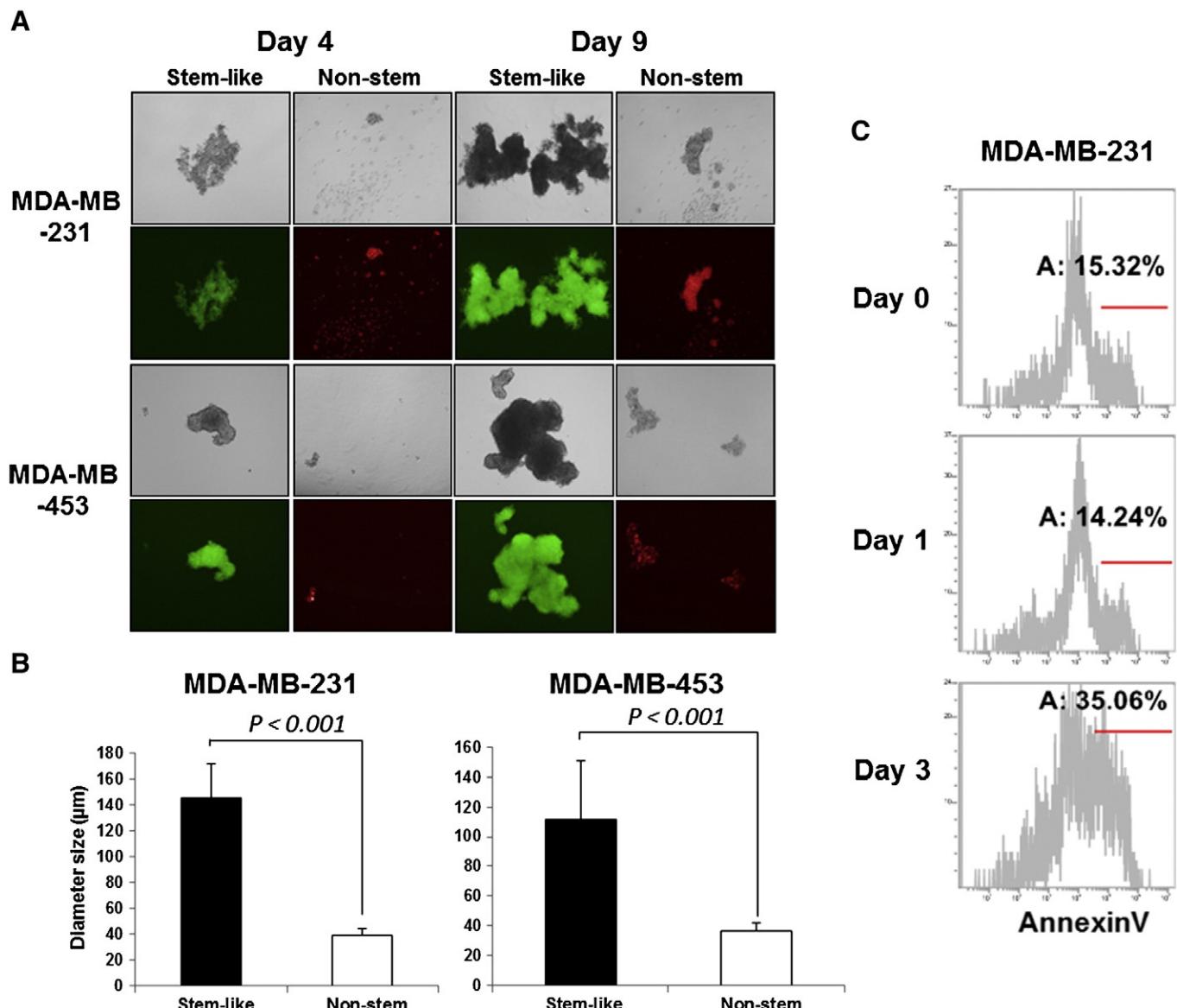
Fig. 2. Mammosphere formation of MDA-MB-231 and MDA-MB-453 CSC-like and non-stem cancer cells. (A) Cells (1000) were plated into ultra-low attachment plates and phase-contrast images (upper panels) or fluorescence images (lower panels) of mammospheres of stem-like (left panels) or non-stem (right panels) cells were obtained 4 days or 9 days later. (B) The numbers and sizes of mammospheres were determined and statistically analyzed. Error bars represent the SD from six samples (p<0.05). (C) Non-stem cells were plated into ultra-low attachment plates. After 1 or 3 days, cells were stained with APC-conjugated annexin V. Apoptosis was detected by the flow cytometric assay. Control: monolayer cultured cells.

3. Results

3.1. Characterization of CSC-like cells and non-stem cells

MDA-MB 231 and MDA-MB-453 CSC-like cells can proliferate without further differentiation and have characteristics of tumor-initiating cells [27]. This property arises as the result of stable transfection of the cells with a human Oct-3/4 promotor driving the expression of GFP, although the mechanism of the block remains unclear. On the other hand, the corresponding non-stem cancer cells were isolated with a plasmid expressing RFP under the control of a CMV immediate early promoter. CSC-like cell and non-stem cell populations can be readily shown to express high levels of GFP and RFP, respectively (Fig. 1A). Fig. 1B shows MDA-MB-231 and MDA-MB-453 CSC-like cells selectively expressed octamer binding transcription factor 3/4 (Oct-4), which is known to maintain CSC-like properties [28]. Fig. 1C shows that unlike non-stem cancer cells, CSC-like cells were highly enriched with CD44+ and CD24− as previously reported [27].

3.2. Mammosphere formation in CSC-like cells and non-stem cells

Stem cells have several unique properties which distinguish them from differentiated cells. One of those is a self-renewal capacity. Gabriela Dontu et al. [29] reported that nonadherent mammospheres were enriched in cells with the functional characteristics of self-renewal potential of stem cells. They developed a strategy that allows for the cultivation of undifferentiated human mammary epithelial cells–mammospheres in suspension like neurospheres. For mammosphere formation assay, MDA-MB-231 and MDA-MB-453 CSC-like cells or non-stem cells were placed in ultra-low attachment 24 well culture plates. CSC-like cells formed mammospheres well, whereas most of the non-stem cells died and only part of the

remaining non-stem cells produced viable mammospheres (Fig. 2A). Mammospheres of CSC-like cells grew faster than those of non-stem cells and the difference of mammosphere size between CSC-like cells and non-stem cells was about 3–4 times 9 days after plating (Fig. 2B). Data from cytometric assay clearly show that most of the non-stem cells died by detachment-induced apoptosis—anoikis (Fig. 2C).

3.3. Xenograft tumor formation in CSC-like cells and non-stem cells

We used a xenograft model of mammary fat pad (both sites of upper mammary fat pad) by subcutaneous injection of CSC-like cells and non-stem cells respectively to assess directly if distinct tumor formation differences were apparent between these cell lines (Fig. 3A). Our observation was that not only CSC-like cells but also non-stem cells formed tumors finally in all ten nude mice. However, the growth rate and final size of the tumors in each population were quite distinct. Fig. 3B shows that the average tumor size from CSC-like cells was 323.5 mm3 and from non-stem cells was 70.4 mm3 (a difference of about 4.5 times). After the mice were sacrificed, we dissected the whole tumor mass and immediately confirmed the fluorescence derived from individual cell lines (Fig. 3C). From CSC-like cells, tumor mass showed green fluorescence, GFP, and from non-stem cells, tumor mass showed red fluorescence, RFP. To determine OCT-4 gene expression in the tumors, we used western blot analysis. As shown in Fig. 3D, as well as the large amount of Oct-4 expressed by tumors of CSC-like cells, a small amount of Oct-4 was expressed by tumors of non-stem cells. From these observations, we hypothesize a dynamic equilibrium theory. We propose that removing cancer stem cells prompts part of the non-stem cell population to convert into CSC-like status during mammosphere formation, similar to what occurred in xenograft tumor.
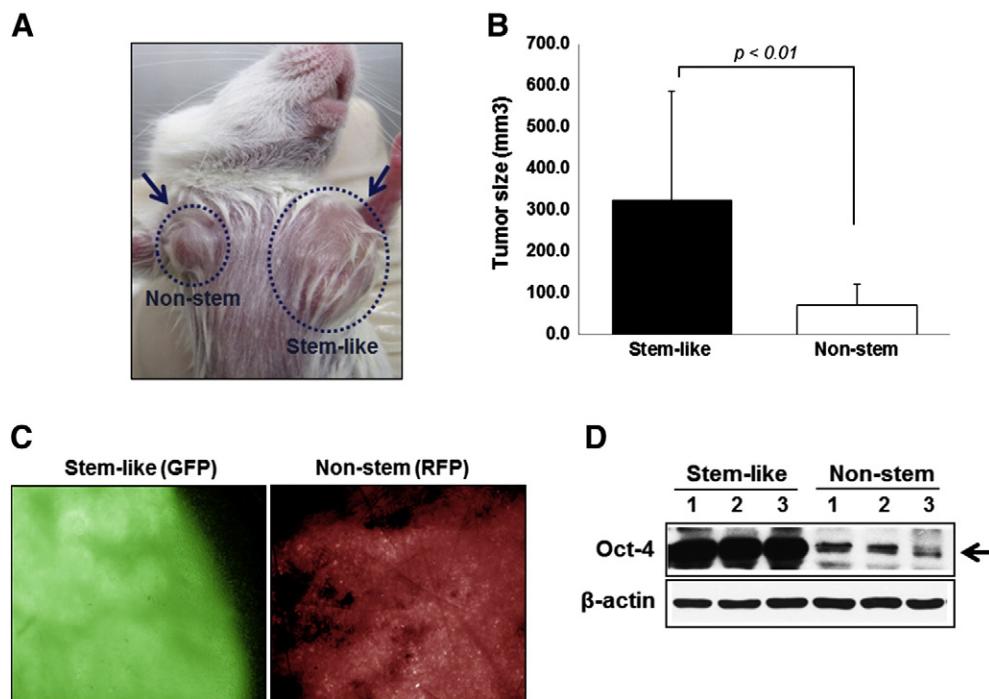
Fig. 3. Comparison of xenograft tumor formation in MDA-MB-231 stem-like cells and non-stem cells. (A) Stem-like (1×10^4) cells and non-stem (1×10^4) cells were injected into the upper mammary fat pad of left and right side, respectively, in NOD/SCID mice (n = 10). (B) Tumor volumes were measured 35 days after injection. The tumor volume showed significant difference (p = 0.01). (C) Tumor was harvested 35 days later and cryosection was performed to detect cells containing GFP (stem-like cells) or RFP (non-stem cells). (D) Lysates containing equal amounts of protein from tumor tissues were separated by SDS-PAGE and immunoblotted with anti-Oct-4 antibody. Actin was shown as an internal standard.

3.4. Conversion of non-stem cells into stem-like cells

To test a dynamic equilibrium hypothesis, we examined whether non-stem cells can convert to stem-like cells during mammosphere formation. Ginestier et al. [30] reported that aldehyde dehydrogenase (ALDH) was increased in a subpopulation of normal and cancerous human mammary epithelial cells which exhibited stem/progenitor cell properties. Data from the Aldefluor assay revealed an increase in ALDH activity in non-stem cells during mammosphere formation. Fig. 4A shows, compared to monolayer cells, a 10 to 11-fold increase in ALDH positive population in mammospheres. These data suggest the presence of CSC-like cell population in mammospheres. This possibility was examined by immunofluorescent staining assay (Fig. 4B). The expression of Oct-4 in the mammosphere was detected in most of the population from MDA-MB-231 and MDA-MB-453 CSC-like cells (both upper panels in Fig. 4B). The expression of Oct-4 was

also detected in part of the non-stem cell population (both lower panels in Fig. 4B). These results suggest that part of the non-stem cells convert to CSC-like cells by inducing OCT-4 gene expression during mammosphere culture.

3.5. Cytokine profiles in CSC-like cells and non-stem cells

Recent studies have revealed that several cytokines such as interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-32 (IL-32) are involved in the interaction between cancer stem cells and their tumor microenvironment [26,31–35]. We examined whether cytokines are involved in the conversion of non-stem cells into CSC-like cells. Cytokine production was compared by using cytokine array assay during monolayer culture and mammosphere culture. As shown in Fig. 5, there is no significant change in CSC-like cells. Unlike in CSC-like cells, secretion of growth-related oncogene (Gro)
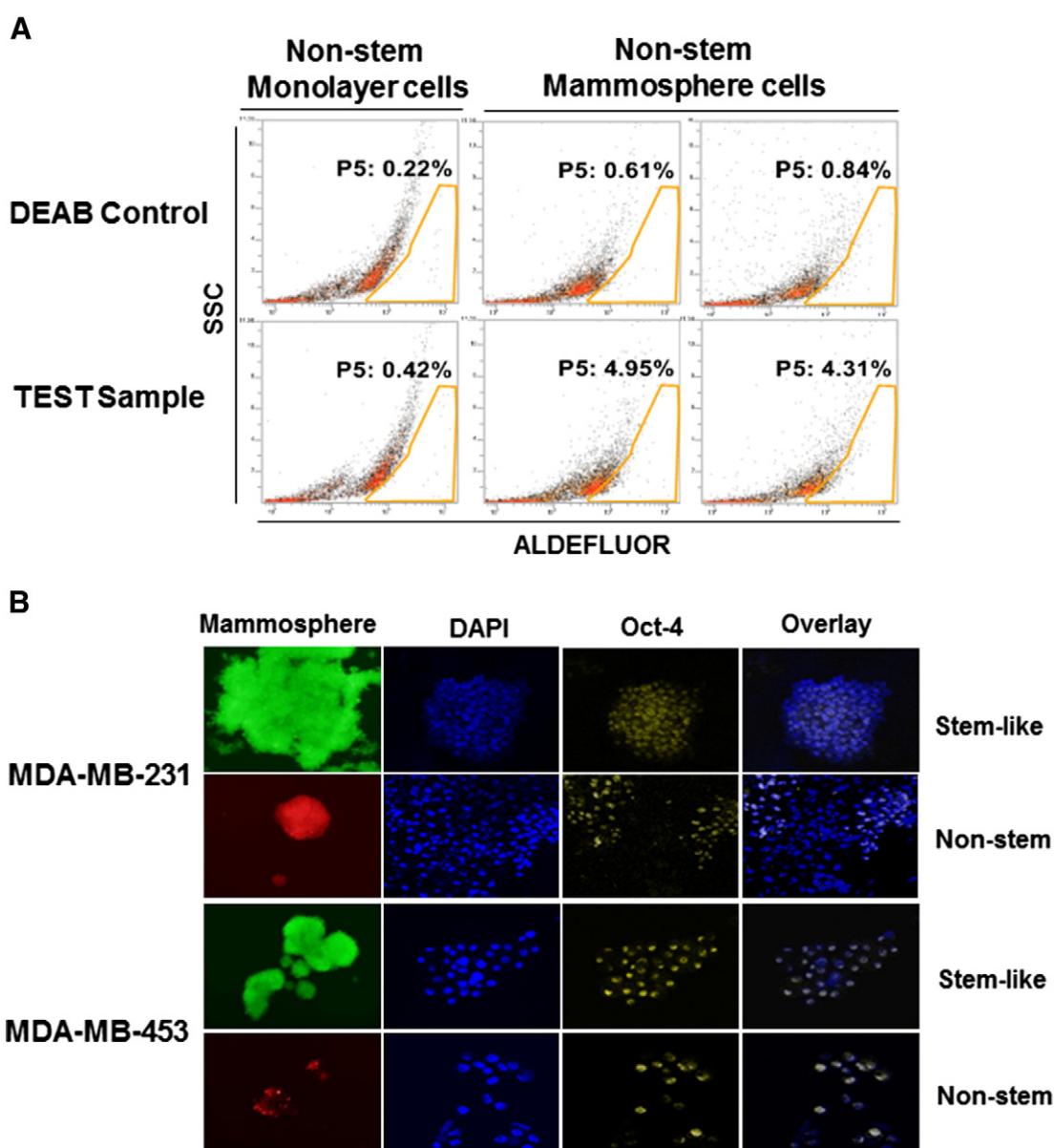
Fig. 4. Aldefluor assay, Oct-4 immunofluorescent staining, and Oct-4 gene expression in mammosphere of non-stem cells. (A) MDA-MB-231 cells from non-stem monolayer culture or duplicate mammosphere cultures (30 days) were labeled with the Aldefluor (BAAA) with and without the ALDH inhibitor DEAB and analyzed with flow cytometry. The numbers shown in each panel reflect the percentage of ALDH + cells in each corresponding group. (B) Mammospheres from MDA-MB-231 and MDA-MB-453 stem-like cells or non-stem cells were cultured for 30 days (left panels), harvested by cytospin, and stained with DAPI or anti-Oct-4 antibody, and then displayed in overlay images.

chemokines (CXCL1, 2, 3) and IL-6 was detected during mammosphere culture in non-stem cells.

3.6. Role of the IL-6-JAK1-STAT3 signal transduction pathway in OCT-4 gene expression in non-stem cells

Previous studies have shown that IL-6 exerts its effects through the JAK1-STAT3 signal transduction pathway [36–40]. We postulated that *OCT-4* gene expression is promoted through the IL-6-JAK1-STAT3 signal transduction pathway. To examine this possibility, non-stem cells were treated with anti-IL-6 antibody during mammosphere culture and the IL-6-JAK1-STAT3-Oct-4 signal transduction pathway was analyzed. Data from immunoblotting assay show that activation (phosphorylation) of JAK1 and STAT3 and increase in Oct-4 expression occurred in non-stem cells during mammosphere culture (Fig. 6A). Treatment with anti-IL-6 antibody inhibited the JAK1 and STAT3 activation as well as *OCT-4* gene expression (Fig. 6B). These data suggest that the IL-6-JAK1-STAT3 signal transduction pathway is involved in the action of *OCT-4* gene expression during mammosphere culture of non-stem cells. These results were confirmed by using STAT3 inhibitors, niclosamide and LLL12. These drugs inhibited STAT3 phosphorylation as well as *OCT-4* gene expression during mammosphere culture (Fig. 7).

4. Discussion

In this study, we observe that part of the non-stem cell population converted to CSC-like status during tumor formation by promoting *OCT-4* gene expression. Non-stem cells, but not CSC-like cells, produced IL-6 which activated the JAK1-STAT3 signal transduction pathway. This autocrine signaling pathway plays an important role

in the conversion of non-stem cells into stem-like cells through upregulation of Oct-4.

Cytokines exert their effects through specific receptors. Various signal transduction pathways are activated through distinct regions of each receptor's cytoplasmic domain. Such pathways include those mediated by the Src (cellular homolog of the Src oncoprotein of Rous sarcoma virus) and JAK (Janus-activated kinase) tyrosine kinase families, STAT (signal transducer and activator of transcription), Smad (Sma and Mad Related Family), MAPK (mitogen-activated protein kinase), and PI3K (phosphatidyl inositol 3 kinase) [41–51]. Among these signaling molecules, STAT proteins play a central role in transmitting cytokine signals [42,52]. In this study, we investigated the IL-6 signal transduction pathway which is known to be activated through the IL-6 receptor. Fig. 6 and previous studies have shown that IL-6 activates the JAK1-STAT3 signal transduction pathway [36–40].

STAT3 is a transcription factor which is encoded by the STAT3 gene [53]. STAT3 is activated through phosphorylation of tyrosine residue 705, which induces homodimerization or heterodimerization with other STAT proteins and results in nuclear translocation and activation of the STAT3 transcriptional regulatory function [54,55]. Phosphorylation of STAT3 can be induced by various cytokines including interferons, IL-5 and IL-6 (Fig. 6 and [56]) and also by receptor and nonreceptor tyrosine kinases such as epidermal growth factor receptor (EGFR) [57,58] and Src [59]. Activation by IL-6 is mediated by members of the JAK kinase family; the tyrosine kinases EGFR and Src can directly phosphorylate STAT3 [60]. STAT3 mediates the expression of a variety of genes including autotaxin, twist, snail, tenascin-C, IL-8, vascular endothelial growth factor (VEGF), survivin, and matrix metalloproteinase-9 (MMP-9) in response to cell stimuli [61–64]. In this study, we observed that STAT3 mediates the expression of Oct-4 (Fig. 6). STAT3-mediated stem cell marker *OCT-4* gene
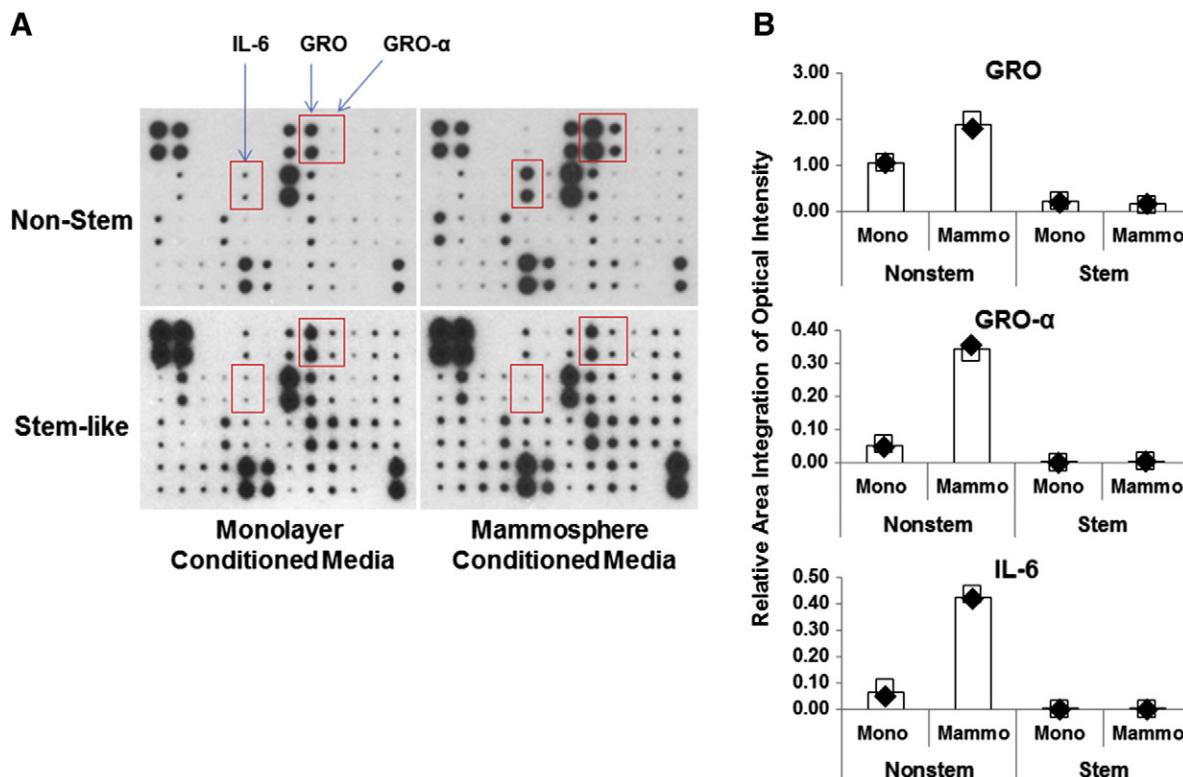
Fig. 5. Comparison of cytokine production in MDA-MB-231 stem-like cells and non-stem cells. (A) Cells were plated in 60-mm petri dishes or ultra-low attachment plates. Conditioned media from monolayer culture or mammosphere culture were harvested and subjected to cytokine antibody arrays. (B) The cytokine array image was analyzed with densitometer and average of area integration of optical intensities for each pair of cytokine spots was plotted.
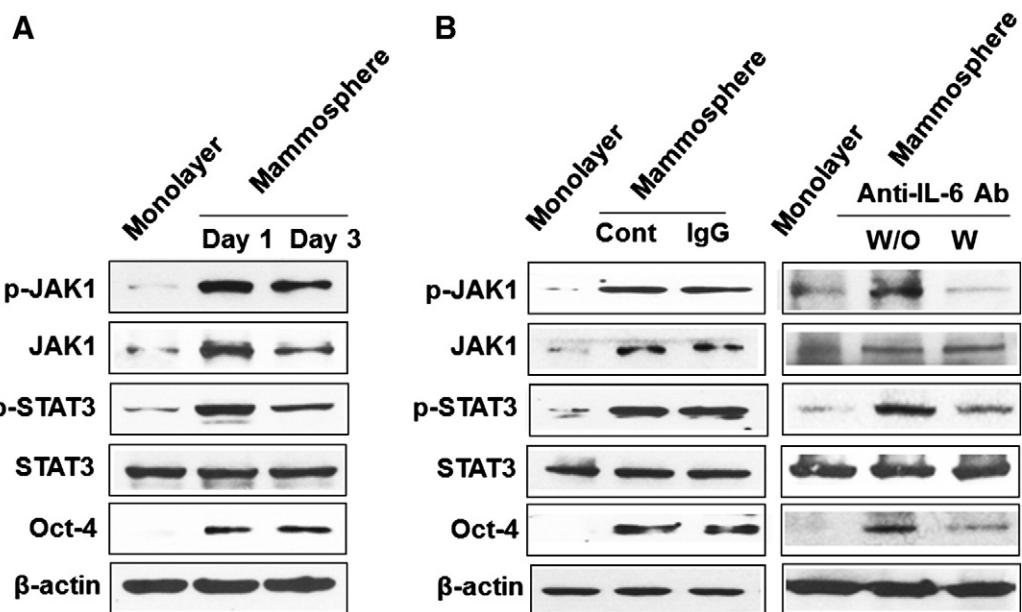
Fig. 6. Effect of anti-IL-6 antibody on the IL-6-JAK1-STAT3-Oct-4 signal transduction pathway in MDA-MB-231 non-stem cells. (A) Cells were cultured in regular plate (monolayer culture) or ultra-low attachment plate (mammosphere culture). After 1 or 3 days, cells were harvested and lysates containing equal amounts of protein were separated by SDS-PAGE and immunoblotted with anti-phospho-JAK1, anti-JAK1, anti-phospho-STAT3, anti-STAT3, or anti-Oct-4 antibody. Actin was shown as an internal standard. (B) Cells were incubated with or without 1 μg/ml anti-IgG as a negative control or 1 μg/ml anti-IL-6 antibody for 1 day in the ultra-low attachment plate for mammosphere culture. Lysates from these cells were assessed by immunoblot analysis.

expression was effectively suppressed by treatment with STAT3 inhibitors, niclosamide or LLL12 (Fig. 7).

Our studies suggest that secreted IL-6 from non-stem cells plays an important role in the conversion of non-CSCs to CSCs through activation of the JAK1-STAT3-Oct-4 signal transduction pathway. An unanswered question is how non-stem cells produce IL-6 during mammosphere culture. At the present time, we can only speculate on the production of IL-6 in non-stem cells. One possibility is that different types of integrin-associated signal transduction pathways such as Notch and Wnt signaling [65] or different levels of integrin-associated proteins such as ILK (integrin-linked kinase), PINCH (particularly interesting Cys-His-rich protein), parvin, and migfilin may exist in CSCs and non-CSCs. Previous studies have shown that among these integrin-associated proteins, migfilin regulates anoikis by influencing Src activation [66]. During mammosphere culture, migfilin is degraded and degradation of migfilin causes Src inactivation which leads to anoikis [66]. Cells of the portion of the non-CSC cells which develop resistance and survive during anoikis may activate Src-p38 MAPK-mediated IL-6 production [67,68]. Obviously, this possibility needs to be examined to understand the mechanism of the conversion of non-CSCs into CSCs.

Author contribution

Seog-Young Kim: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing.

Jin Wook Kang: Collection and assembly of data, data analysis and interpretation.

Xinxin Song: Collection and assembly of data.

Bo Kyoung Kim: Collection and assembly of data.

Young Dong Yoo: Collection and assembly of data.

Yong Tae Kwon: Conception and design, financial support.

Yong J. Lee: Conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

Acknowledgments
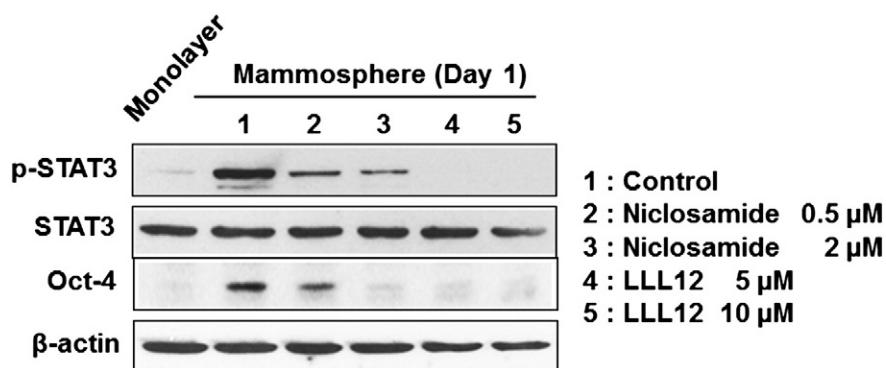
Fig. 7. Effect of STAT3 inhibitors on STAT activation and *OCT-4* gene expression in MDA-MB-231 non-stem cells. Cells were treated with niclosamide (0.5, 2 μM) or LLL12 (5, 10 μM) for 1 day. Lysates containing equal amounts of protein were separated by SDS-PAGE and immunoblotted with anti-phospho-STAT3, anti-STAT3, or anti-Oct-4 antibody. Actin was shown as an internal standard.

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BIOGRAPHICAL

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EDUCATION and TRAINING
List entries in each section chronologically

UNDERGRADUATE:

Dates Attended	Name and Location of Institution	Degree Received and Year	Major Subject
1971-1975	Seoul National University Seoul, Korea	B.S., 1975	Zoology

GRADUATE:

Dates Attended	Name and Location of Institution	Degree Received and Year	Major Advisor and Discipline
1978-1981	University of Illinois, Urbana, Illinois	M.S., 1981	Dr. H.S. Ducoff, Physiology
1981-1984	University of Illinois, Urbana, Illinois	Ph.D., 1984	Dr. H.S. Ducoff, Physiology

POSTGRADUATE:

Include internships, residencies, fellowships and/or any other professional training experience

Dates Attended	*Name and Location of Institution*	*Name of Program Director and Discipline*
1984-1987	University of California San Francisco, California	Dr. W.C. Dewey Radiation Biology

APPOINTMENTS and POSITIONS

ACADEMIC:

Years Inclusive	*Name and Location of Institution of Organization*	*Rank/Title* **Visiting Prefix must be used if faculty member has been appointed pending committee review or position approval*
1977-1978	Seoul National University Seoul, Korea	Teaching Assistant
1978-1983	University of Illinois Urbana, Illinois	Teaching/Research Assistant
1988-1999	Oakland University Rochester, Michigan	Adjunct Assistant/Associate Professor
1999- 2000	University of Pittsburgh Pittsburgh, Pennsylvania	Visiting Associate Professor Department of Pharmacology
2000-2005	University of Pittsburgh Pittsburgh, Pennsylvania	Associate Professor Department of Pharmacology
2002-2005	University of Pittsburgh Pittsburgh, Pennsylvania	Associate Professor Department of Surgery
2005-Present	University of Pittsburgh Pittsburgh, Pennsylvania	Professor Department of Surgery and Pharmacology & Chemical Biology (secondary appointment)

NON-ACADEMIC:

Include military or other Government service

Years Inclusive	Name and Location of Institution of Organization	Rank/Title Or Position
1975-1977	*The 3rd Military Academy* *Korea*	Second/First Lieutenant Instructor for biology program
1987-1999	*William Beaumont Hospital* *Radiation Oncology* *Royal Oak, Michigan*	Biophysicist/Molecular Biologist

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CERTIFICATION and LICENSURE

SPECIALTY CERTIFICATION:

Certifying Board	*Year*
N/A	

MEDICAL or OTHER PROFESSIONAL LICENSURE:

Licensing Board/State	*Year*
N/A	

MEMBERSHIPS in PROFESSIONAL and SCIENTIFIC SOCIETIES

Organization	*Year*
Radiation Research Society	1984
North American Hyperthermia Society	1984
American Association for Cancer Research	1990
American Association for the Advancement of Science	1995
Federation of American Society for Experimental Biology	2002

HONORS

Title of Award	*Year*
University Scholarship Award	1972-1973
University Fellowship Award	1974
Graduation with honors	1975
Radiation Oncology Traineeship Award	1983
Radiation Research Society Travel Award	1987

PUBLICATIONS
List separately the following categories and use citation format of current INDEX MEDICUS

1.   Refereed articles

1. **Lee, Y.J.,** and Ducoff, H.S., Age and Sensitivity to Oxygen in the Flour Beetles, *Tribolium confusum.* Mech. Aging Dev., 21, 97-103, 1983.

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3. **Lee, Y.J.** and Dewey, W.C., Protection of Chinese Hamster Ovary Cells from Hyperthermic Killing by

Cycloheximide or Puromycin. Radiat. Res., 106, 98-110, 1986.

4. **Lee, Y.J.** and Dewey, W.C., Effect of Cycloheximide or Puromycin on Induction of Thermotolerance by Sodium Arsenite in Chinese Hamster Ovary Cells: Involvement of Heat Shock Proteins. J. Cell. Physiol., 132, 41-48, 1987.

5. **Lee, Y.J.,** Dewey, W.C., and G.C. Li, Protection of Chinese Hamster Ovary Cells from Heat Killing by Treatment with Cycloheximide or Puromycin: Involvement of HSPs. Radiat. Res., 111, 237-253, 1987.

6. **Lee, Y.J.**, and Dewey, W.C., Induction of Heat Shock Proteins in Chinese Hamster Ovary Cells and Development of Thermotolerance by Intermediate concentration of Puromycin. J. Cell. Physiol., 132, 1-11, 1987.

7. **Lee, Y.J.** and Dewey, W.C., Effect of Cycloheximide or Puromycin on Induction of Thermotolerance by Heat in Chinese Hamster Ovary Cells: Dose Fractionation at 45.5°C. Cancer Res., 47, 5960-5966, 1987.

8. **Lee, Y.J.** and Dewey, W.C., Thermotolerance Induced by Heat, Sodium Arsenite, or Puromycin: Its Inhibition and Differences between 43°C and 45°C. J. Cell. Physiol., 135, 397-406, 1988.

9. Armour, E.P., **Lee, Y.J.,** Corry, P.M., and Borrelli, M.J., Protection from Heat-Induced Protein Migration and DNA Repair Inhibition by Cycloheximide. Biochem. Biophys. Res. Commun., 157, 611-617, 1988.

10. **Lee, Y.J.** and Ducoff, H.S., Radiation Factors and Their Influence on Induction of Oxygen Resistance. Radiat. Res., 117, 158-162, 1989.

11. Chopp, M., Tidwell, C.D., **Lee, Y.J**., Knight, R., Helpern, J.A., and Welch, K.M.A., Reduction of Hyperthermic Ischemic Acidosis by a Conditioning Ischemic Event in Cats. Stroke, 20, 1357-1360, 1989.

12. **Lee, Y.J.,** Armour, E.P., Borrelli, M.J., and Corry, P.M., Heat Protectors and Heat-Induced Redistribution of 70 and 26 kDa Proteins in Chinese Hamster Ovary Cells. J. Cell. Physiol., 141, 510-516, 1989.

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14. **Lee, Y.J**., Perlaky, L., Dewey, W.C., Armour, E.P., and Corry, P.M., Differences in Thermotolerance Induced by Heat or Sodium Arsenite: Cell Killing, and Protein Synthesis Inhibition. Radiat. Res., 121, 295-303, 1990.

15. **Lee, Y.J.**, Kim, D., and Corry, P.M. Effect of Histidine on Histidinol-Induced Heat Protection in Chinese Hamster Ovary Cells. J. Cell. Physiol., 144, 401-407, 1990.

16. **Lee, Y.J.,** Hou, Z.Z., Curetty, L., Borrelli, M.J., and Corry, P.M., Correlation between Redistribution of 26 kDa Protein and Development of Chronic Thermotolerance in Various Mammalian Cell Lines. J. Cell. Physiol., 145, 324-332, 1990.

17. **Lee, Y.J.,** Curetty, L., and Corry, P.M. Heat-Induced Preferential Synthesis and Redistribution of HSP 70 and 28 Families in Chinese Hamster Ovary Cells. Biochem. Biophys. Res. Commun., 172, 119-125, 1990.

18. **Lee, Y.J.,** Kim, D., Hou, Z., and Corry, P.M. Differences in Thermotolerance Induced by Heat or Sodium Arsenite: Correlation between Redistribution of a 26 kDa protein and Protein Synthesis-Independent Thermotolerance Development in CHO cells. Radiat. Res., 127, 325-334, 1991.

19. **Lee, Y.J.,** Dewey, W.C., and Marton, L.J. Mechanisms of Heat Protection by Cycloheximide or Puromycin:Involvement of Polyamines? J. Therm. Biol., 16, 377-379, 1991.

20. **Lee, Y.J.,** Borrelli, M.J., and Corry, P.M. Mechanism(s) of Heat Killing: Accumulation of Nascent Polypeptides in the Nucleus? Biochem. Biophys. Res. Commun., 176, 1525-1531, 1991.

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43. Kim, S.H., Kim, J.H., Erdos, G., and **Lee, Y.J.** Effect of Staurosporine on Suppression of Heat Shock Gene Expression and Thermotolerance Development in HT-29 Cells. Biochem. Biophys. Res. Commun., 193, 759-763, 1993.

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46. **Lee, Y.J.,** Hou, Z., Erdos, G., Cho, J.M., and Corry, P.M. Homoharringtonine Induces Heat Protection and Facilitates Dissociation of HSF-HSE Complex During Heating at 43°C in HT-29 Cells. Biochem. Biophys. Res. Commun., 197, 1011-1018, 1993.

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53. **Lee, Y.J.,** Kim, J.H., Ryu, S., Kim, S.H., Vidair, C.A., Armour, E.P., and Corry, P.M. Mechanisms of Mild Hyperthermia-Induced Cytotoxicity in Human Prostatic Carcinoma Cells: Perturbation of Cell Cycle Progression and DNA Fragmentation. J. Therm. Biol., 19, 305-313, 1994.

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56. **Lee, Y.J.,** Berns, C.M., Erdos, G., Borrelli, M.J., Ahn, C.H., and Corry, P.M. Effect of 1-(5-Isoquinolinesulfonyl)-2-Methylpiperazine (H-7) on HSP70 and HSP28 Gene Expression and Thermotolerance Development in Human Colon Carcinoma Cells. Biochem. Pharmacol., 48, 2057-2063, 1994.

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60. Stromberg, J.S., **Lee, Y.J.,** Armour, E.P., Martinez, A.A., and Corry, P.M. Lack of Radiosensitization after Paclitaxel Treatment of Three Human Carcinoma Cell Lines. Cancer, 75, 2262-2268, 1995.

61. Erdos, G., **Lee, Y.J.,** Cho, J.M., and Corry, P.M. Heat-Induced bFGF Gene Expression in the Absence of Heat Shock Element Correlates with Enhanced AP-1 Binding Activity. J. Cell. Physiol., 164, 404-413, 1995.

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94. Kim, J.H., and **Lee, Y.J.** Low glucose enhances TRAIL-induced reduction of Akt expression: Involvement of caspase-3. Presented at the 49th Annual Meeting of the Radiation Research Society in Reno, April 20-24. 2002.

95. Song, J., Rice, P., and **Lee, Y.** Role of Daxx in ASK1-SEK1-JNK1 Signal Trafficking During Metabolic Oxidative Stress. Presented at the 14th Annual Scientific Retreat of the University of Pittsburgh Cancer Institute, December 12-13, 2002

96. Song, J.J., and **Lee, YJ.** Role of glutaredoxin in metabolic oxidative stress. Presented at the 94th Annual Meeting of the American Association for Cancer Research in Washington, DC, July 11-14, 2003.

97. Rhee, J.G., Song, J.J., **Lee, Y.J.,** and Shibata, T. Replication Capability of an Engineered Adenoviral Vector in Hypoxic Conditions. Presented at ICRR, August 17-22, 2003.

98. **Lee, Y.J.,** and Song, J.J. Role of the ASK1-SEK1-JNK1-HIPK1 signal in Daxx trafficking and ASK1 oligomerization. Presented at The Sixth AACR-Japanese Cancer Association Joint Conference Advances in Cancer Research in Waikoloa, Hawaii, January 25-29, 2004.

99. Song J.J., and **Lee, YJ.** Role of the ASK1-SEK1-JNK1-HIPK1 signal in Daxx trafficking. Presented at the 95th Annual Meeting of the American Association for Cancer Research in Orlando, March 27-31, 2004.

100. Song J.J. and **Lee, Y.J.** Tryptophan 621 and serine 667 residues of Daxx regulate its nuclear export during oxidative stress. Presented at the 51st Annual Meeting of the Radiation Research Society in St. Louis, Missouri, April 24-27, 2004.

101. Song, J.J. and **Lee, Y.J.** Dissociation of Akt1 from its negative regulator JIP1 is mediated through the SEK1-JNK2 signal transduction pathway during glucose deprivation: a negative feedback loop. Presented at the 96th Annual Meeting of the American Association for Cancer Research in Anaheim, Orange County, CA, April 16-20, 2005.

102. Kim, K.M. and **Lee, Y.J.** Amiloride augments TRAIL-induced apoptotic death by inhibiting phosphorylation of kinases and phosphatases associated with the PI3K-Akt pathway. Presented at the 96th Annual Meeting of the American Association for Cancer Research in Anaheim, Orange County, CA, April 16-20, 2005.

103. Lew, K.L., Srivastava, S.K., Choi, S., Xiao, D., Zeng, Y., Johnson, C.S., Trump, D.L., **Lee, Y.J.**, and Singh, S.V. Sulforaphane-induced cell death in human prostate cancer cells is initiated by generation of reactive oxygen species. Presented at the 96th Annual Meeting of the American Association for Cancer Research in Anaheim, Orange County, CA, April 16-20, 2005.

104. Song, J.J. and **Lee**, Y.J. Dissociation of Akt1 from its negative regulator JIP1 is mediated through the SEK1-JNK2 signal transduction pathway during glucose deprivation: a negative feedback loop. Presented at the 3rd Annual Department of Surgery Research Day, Pittsburgh, PA, April 27, 2005.

105. Kim, K.M. and **Lee, Y.J.** Role of HER-2/neu Signaling in Sensitivity to Tumor Necrosis Factor-related Apoptosis-inducing Ligand: Enhancement of TRAIL-mediated Apoptosis by Amiloride. Presented at the 3rd Annual Department of Surgery Research Day, Pittsburgh, PA, April 27, 2005.

106. Song, J.J. and **Lee, Y.J.** Dissociation of Akt1 from its negative regulator JIP1 is mediated through the SEK1-JNK2 signal transduction pathway during glucose deprivation: a negative feedback loop. Presented at the 13th Annual Department of Pharmacology Retreat, Seven Springs Mountain Resort, April 29, 2005.

107. Kim, K.M. and **Lee, Y.J.** Role of HER-2/neu Signaling in Sensitivity to Tumor Necrosis Factor-related Apoptosis-inducing Ligand: Enhancement of TRAIL-mediated Apoptosis by Amiloride. Presented at the 13th Annual Department of Pharmacology Retreat, Seven Springs Mountain Resort, April 29, 2005.

108. Kwon, S.J. and **Lee, Y.J.** Effect of Low Glutamine/Glucose on Hypoxia-induced Elevation of HIF-1α in Human Pancreatic Cancer MiaPaCa-2 and Human Prostatic Cancer DU-145 cells. Presented at the 52nd Annual Meeting of the Radiation Research Society in Denver, Colorado, October 16-19, 2005.

109. Rhee, J.G., Gu, Y.H., and **Lee, Y.J.** Uneven distribution of green fluorescence positive cells within multicell spheroids following adenoviral transduction. Presented at the 52nd Annual Meeting of the Radiation Research Society in Denver, Colorado, October 16-19, 2005.

110. Ziauddin, M. Firdos, Guo, Z., Popovic, P., Kavanagh, M., O'Malley, M., **Lee, Y.J.**, and Bartlett, D.L. Improved in vitro cytotoxicity of vaccinia virus in colon cancer cells by expression of murine tumor necrosis factor-related apoptosis-inducing ligand and combination with chemotherapy. Presented at the 97th Annual Meeting of the American Association for Cancer Research in Washington, DC, April 1-5, 2006.

111 Song, J.J., and **Lee, Y.J.** Cross-talk Between JIP3 and JIP1 During Glucose Deprivation. Presented at the 97th Annual Meeting of the American Association for Cancer Research in Washington, DC, April 1-5, 2006.

112. Lee, Y.J. and Jae J. Song Differential activation of the JNK signal pathway by UV irradiation and metabolic oxidative stress. Presented at the 53nd Annual Meeting of the Radiation Research Society in Philadelphia, Pennsylvania, November 5-8, 2006.

113. Sun, B.K., Kim, J.H., Oh, S., Kim, S.Y., Choi, H.J., **Lee, Y.J.**, and Song, J.J. TRAIL-induced p38 activation is regulated by MEKK4 with positive feedback manner. Presented at the 102nd Annual Meeting of the American Association for Cancer Research in Orlando, FL, April 2-6, 2011.

114. Kim, K.M. and **Lee, Y.J.** Effect of hyperthermia on apoptotic death induced by TRAIL or mapatumumab. Presented at the 9th Annual Department of Surgery Research Day, Pittsburgh, PA, May 18, 2011.

115. Kim, S.Y., Pronchownik E.V. and **Lee, Y.J.** Permanently blocked cancer stem cell-like cells are sensitive to ionizing radiation. Presented at the 9th Annual Department of Surgery Research Day, Pittsburgh, PA, May 18, 2011.

116. Song, X., Kim, H., Kim, S., Basse, P., Park, B., Lee, B., **Lee, Y.J.**'Hyperthermia-Enhanced TRAIL- And Mapatumuab-Induced Apoptosis Is Mediated Through Loss of Mitochondrial Membrane Potential. First

Regional Translational Research in Mitochondria, Aging, and Disease –TrMAD Symposia, Pittsburgh, October 29, 2011.

117. Kim, S.Y., Pronchownik E.V., Spitz, D., and **Lee, Y.J.** Breast cancer stem cell-like cells are more sensitive to ionizing radiation than non-stem cells: role of ATM. Presented at the 103rd Annual Meeting of the American Association for Cancer Research in Chicago, IL, March 31-April 4, 2012.

118. Song, X., Kim, S., and **Lee, Y.J.** The Role of Bcl-xL in Synergistic Induction of Apopotosis by Mapatumumab and Oxaliplatin in Combination with Hyperthermia on Human Colon Cancer. Second Regional Translational Research in Mitochondria, Aging, and Disease –TrMAD Symposia, Pittsburgh, October 20, 2012.

119. Kim, S.Y., Kang, J.W., Song, X., Kim, B., Yoo, Y.D., Kwon, Y.T. and **Lee, Y.J.** Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. Presented at the 104th Annual Meeting of the American Association for Cancer Research in Washington DC, April 6-April 10, 2013.

4. **Other publications**

PROFESSIONAL ACTIVITIES

TEACHING:

Provide a summary of courses and tutorials taught (include numbers and types of students taught, contact hours, number of lectures, etc), other lectures and seminars given, theses completed under your direction, service on Ph.D. committees, supervision of pre- and post-doctoral students, house staff physicians and fellows (clinical and research), specialty board memberships, continuing medical education activities.

Teaching: Introductory biology course (1975-1977), introductory human physiology (1978-1980), general physiology (1981), introductory human physiology (1982-1983), radiation biology (1987-1999; 1 lecture/yr), integrated case studies course (2000: discussion, nine medical students for 22 hrs), introduction to being a physician (2001: discussion, nine medical students for 8 hrs), introduction to being a physician (2002: discussion, nine medical students for 15.5 hrs), radiation biology (2003: 4 lectures; three residents for 4 hrs), introduction to being a physician (2003: discussion, nine medical students, 15 hrs scheduled), radiation biology (2004: 3 lectures; four residents for 3 hrs), radiation biology (2005: 3 lectures; four residents for 3 hrs), radiation biology (2006: 3 lectures; two residents for 3 hrs), radiation biology (2007: 3 lectures; two residents for 3 hrs), radiation biology (2008: 3 lectures; four residents and one medical physicist for 3 hrs), radiation biology (2009: 3 lectures; four residents and one medical physicist for 3 hrs), radiation biology (2010: 3 lectures; four residents for 3 hrs), radiation biology (2011: 3 lectures; five residents for 3 hrs)......

Postdoctoral fellows/research associates: Twenty nine postdoctoral research fellows were/are being supervised: Lidija Timcenko, Ph.D. (November 1988 - April 1989), Dooha Kim, Ph.D. (April 1989 - August 1992), Zi-Zheng Hou, M.D.(August 1989 - December 1993), Zhe S. Piao, M.D., Ph.D. (February 1991 - October 1991), Geza

Erdos, Ph.D. (July 1992 - August 1995), Richard Y. Liu, M.D. (January 1993 - March 1994), Robert Blackburn, Ph.D. (June 1994 - February 1999), Xin Liu, Ph.D. (February 1996 - May 1997), Kun H. Lee (March 1999-February 2000), Seon Y. Nam (January 2000- April, 2001), Young K. Song (March 2000- September 2002), Jin Kim (February 2001-March 2003), Jae J. Song (March 2001-January 2009), Ki M. Kim (October 2003-June 2005), Seok J. Kwon (January 2004-November 2004), Seung C. Yang (January 2004- October 2004), Young H. Kim (December 2004-June 2007), Joon S. Song (December 2004-June 2005), Young M. Yu (February 2005-December 2005), Jinsang Yoo (October 2005-September 2007), Byoung K. Yoo (April 2006-October 2006), Seong C. Kim, Ph.D. (April 2006-September 2007), Jae-Hoon Jeong (August 2006-July 2008), Dae-Hee Lee (August 2006-October 2008), Sung S. Park (November 2006-July 2008), Guang Jin (February 2009- July 2009), Kyung Soo Park (September 2009-April 2010), Seog-Young Kim (August 2010-present), Han-Cheon Kim (August 2010-June 2011), Xinxin Song (August 2011-present).

Residents: Two residents were trained: Jannifer S. Stromberg, M.D.(July 1992 - June 1993), and Anjali Gupta, M.D.   (July 1995 - June 1996) and one surgical oncology fellow: Marco A. Alcala, M.D. (January 2009-June 2010).

Visiting scholars: Byung S. Cho, M.D. (July 1994 - September 1994), Kyungjin Kim, Ph.D. (January 1997 - February 1997), Sun H. Baek, Ph.D. (June 2000 – August 2000), Seung-Hyun Kwon, B.S. (March 2003- June 2003), Mi-Sun Moon, M.S. (November 2003- June 2004).

Volunteers and work study students: Harris Vuaden(September 1999-January 2001), Jason McIntyre (September 2000-May 2002), Svetlana Toshniwal, M.D. (September 2000- December 2000), Xiaoning Liu, M.D. (October 2000- January 2001), Angelica Leon, M.S. (February 2001-August 2001), Safia Sulaiman (February 2001-June 2001), Mubasshir Ajaz (February 2001-November 2002), Deborah Newman-Williams (October 2001-December 2001), Geeta Godara (September 2002-December 2002), Heather Herman (October 2002-May 2003), Jason Pascual (November 2002-May 2003), Jeong-Ah Kwon (November 2002-February 2003), Sally Morton (September 2003- July 2004), Michelle Pasquino (August 2004-May 2005), Cliff Kim (June 2007-August 2007), Bokyoung Kim (July 2012-August 2012).

Committee activity: 2002 Cancer Education Program, University of Pittsburgh Cancer Institute
 2003-present Glassware Facility Committee at Hillman Cancer Center
 PO1 Evaluation Committee for Dr. Dennis Leeper at the Department of Radiation
 Oncology, Thomas Jefferson University
 2007-present Ph.D. Thesis committee (2012: Sung Tae Kim)

Seminars:

1. Invited Seminarian, Seoul National University, Seoul, Korea, June 1986.

2. Invited Seminarian, Oakland University, Rochester, Michigan, November 1987.

3. Invited Seminarian, Seoul National University, Seoul, Korea, August 1988.

4. Invited Seminarian, Kyung Hee University, Seoul, Korea, August 1988.

5. Invited Seminarian, UCSF, San Francisco, California, May 1989.

6. Invited Seminarian, University of Iowa, Iowa City, Iowa, October 1989.

7. Role of HSP70 in Thermotolerance Development, Henry Ford Hospital, Detroit, Michigan, November 1991.

8. Physiological and Biochemical Functions of HSP70, UCSF, San Francisco, California, March 1992.

9. Potentiation of Cytokine Cytotoxicity by Heat or Drug, Lucky Biotech. Emeryville, California, March 1992.

10.The Possible Role of HSP70 in Heat-Induced Cytotoxicity, Wayne State University, Detroit, Michigan, March 1992.

11.Application of Genetic Engineering in Thermobiology, Div. of Gastroenterology-Hepatology, William Beaumont Hospital, Royal Oak, Michigan, April 1993.

12.How Does Tumor Grow? The Role of AP-1 Transcription Factors in bFGF Gene Expression, Seoul National University, Seoul, Korea, October 1995.

13. Protooncogene, Angiogenesis and Apoptosis in Human Tumor Cell Lines, LG Chem, Biotech Research Institute, Taejon, Korea, October 1995.

14. How Does Tumor Grow? Environment Stresses-Induced Angiogenic Factors, Kang Lung University, Kang Lung, Korea, October 1995.

15.The Role of AP-1 Binding Factors in the Regulation of bFGF Gene Expression in Human Carcinoma Cells, Henry Ford Hospital, Detroit, Michigan, November 1995.

16. Involvement of AP-1 Transcription Factors in bFGF Gene Expression, Department of Pharmacology, Wayne State University, Michigan, May 1997.

17. Stress Responses in Tumor Cells and Its Application in Targeted Gene Therapy, College of Medicine, Seoul National University, Seoul, Korea, October 1997.

18. Stress Responses in Tumor Cells and Its Application in Targeted Gene Therapy,   K-jist,   Kwang-Ju, Korea October 1997.

19. Stress Responses in Tumor Cells and Its Application in Targeted Gene Therapy,   LG Chem, Tajeon, Korea, October 1997.

20. Glucose Deprivation-induced Oxidative Stress, Signal Transduction, Duksung Women's University, Seoul, Korea, March 1998.

21. Metabolic Oxidative Stress Responses in Tumor Cells, Inchon University, Inchon, Korea, April 1998.

22. Stress Responses in Tumor Cells: Glucose Deprivation-induced Signal Transduction, KIST, Taejon, Korea, April 1998.

23. Oxidative Stress-Induced Signal Transduction and Angiogenic Factor Gene Expression, Seoul Joong Ang Hospital, Seoul, Korea, April 1998.

24. Metabolic Oxidative Stress-induced Signal Transduction and Gene Expression, Kyung Hee University, Medical College, Department of Neurology,Seoul, Korea, May 1998.

25. Application of Dual Suicide Gene in Targeted Gene Therapy, Inchon University, Inchon, Korea, May 1998.

26. Application of Dual Suicide Gene in Targeted Gene Therapy, Yonsei Cancer Center, Seoul, Korea, May 1998.

27. Metabolic Oxidative Stress-induced Signal Transduction and Gene Expression, Korea Atomic Energy Research Institute, Seoul, Korea, June 1998.

28. Application of Dual Suicide Gene in Targeted Gene Therapy, Kyung Hee University, Seoul, Korea, June 1998.

29. Oxidative Stress, Signal Transduction, and Gene Expression, Mayo Clinic, Scottsdale, Arizona, July 1998.

30. Metabolic Oxidative Stress-induced Signal Transduction and Gene Expression, Pusan National University, Pusan, Korea, September 1998.

31. Stress responses in tumor cells: metabolic oxidative stress-induced signal transduction and its application in gene therapy, University of Michigan, Ann Arbor, Michigan, September 1998.

32. Metabolic oxidative stress-induced signal transduction , University of Illinois, Uraba-Champaign, Illinois, September 1998.

33. Metabolic oxidative stress-induced signal transduction and its application in gene therapy, University of Pittsburgh, Pittsburgh, Pennsylvania, October 1998.

34. Stress-induced signal transduction and targeted thermo-gene therapy, University of Texas, San Antonio, October 1999

35. Studies on tumor microenvironment: from signal transduction to gene therapy, Wake Forest University, Winston-Salem, North Carolina, October 2000

36. Targeted radio-gene therapy, SUNY Upstate Medical University, Syracuse, New York, April 5, 2001

37. Modulation of TRAIL-induced cytotoxicity, Henry Ford Hospital, Detroit, Michigan, April 17, 2001

38. Role of tumor microenvironment in signal transduction and angiogenesis, Georgetown University, Washington D.C., December 5, 2001

39. Targeted gene therapy, Washington University, St Louis, Missouri, January 10, 2002

40. Tumor microenvironment, signal transduction, and targeted gene therapy, Wake Forest University, Winston-Salem, North Carolina, February 7, 2002

41. Modulation of TRAIL-induced cytotoxicity in prostate cancer, University of Pittsburgh, PUCP, Pittsburgh, Pennsylvania, July 15, 2002

42. Metabolic oxidative stress-induced signal transduction and its application in gene therapy, Roswell Park Cancer Institute, Buffalo, New York, December 2, 2002

43. Metabolic oxidative stress-induced ASK1-SEK1-JNK1 signal transduction and is role in Daxx trafficking. University of Pittsburgh, UPCI, Pittsburgh, Pennsylvania, January 7, 2003

44. Targeted radio-gene therapy, University of California at Davis, Sacramento, California, May 29, 2003

45. Metabolic Oxidative Stress-induced ASK1-SEK1-JNK1-HIPK1 Signal Transduction and Its role in Daxx trafficking, University of Kentucky, Lexington, Kentucky, November 13, 2003

46. Cell signaling and molecular targeting therapy, Southern Illinois University, Springfield, Illinois, January 5, 2004

47. Metabolic Oxidative Stress-induced ASK1-SEK1-JNK1-HIPK1 Signal Transduction and Its role in Daxx trafficking, University of Pittsburgh, Department of Pharmacology, Pittsburgh, Pennsylvania, March 19, 2004

48. Metabolic Oxidative Stress-induced ASK1-MEK-MAPK Signal Transduction and Its role in Daxx Trafficking and JIP Scaffolding, University of Arkansas, Little Rock, Arkansas, October 11, 2004.

49. Metabolic Oxidative Stress-induced ASK1-MEK-MAPK Signal Transduction and Its role in Daxx Trafficking and JIP Scaffolding, Texas A&M University, Temple, Texas, October 15, 2004.

50. Cell Signaling and Targeted Molecular Therapy, The Cancer Institute of New Jersey, University of Dentistry and Medicine New Jersey, New Brunswick, New Jersey, December 13, 2004

51. Regulation of Tumor Microenvironment-induced ASK1-MEK-MAPK Signal Transduction, University of South Florida, Tampa, Florida, March 22, 2005

52. Regulation of Tumor Microenvironment-induced ASK1-MEK-MAPK Signal Transduction, University of California, Irvine, California, April 20, 2005

53. Regulation of Metabolic Oxidative Stress-induced ASK1-MEK-MAPK Signal Transduction, Targeting Oxidative Stress and Signaling Symposium, King of Prussia, Pennsylvania, December 2, 2005

54. Regulation of Metabolic Oxidative Stress-induced ASK1-MEK-MAPK Signal Transduction, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania, June 6, 2006

55. Effects of Quercetin on Cancer: How to Apply Clinically? University of Pittsburgh, Department of Surgery, Pennsylvania, February 7, 2007

56. Effects of Quercetin on Cancer: Apoptosis. University of Pittsburgh Cancer Institute, DAMPs and Cell Death meeting, May 4, 2007

57. Flavonoids and Cancer Prevention. University of Pittsburgh Cancer Institute, Biochemoprevention group meeting, May 9, 2007

58. Flavonoids for Cancer Prevention and Therapy. Louisiana State University, June 6, 2007

59. Tumor Microenvironment-induced Signal Transduction. University of Pittsburgh, Urology, November 5, 2007.

60. Tumor Microenvironment-induced Signal Transduction. West Virginia University, November 27, 2007.

61. Oxidative Activation of the Wogonin-induced p53 Dependent Apoptotic Pathway. University of Pittsburgh, Surgery, April 1, 2009.

RESEARCH:

		Role in Project		
1. **Grant Number**		**and**	**Years**	
Source	**Grant Title**	**Percentage of Effort**	**Inclusive**	**$ Amount**

Currently Funded Grants

National Institutes of Health NIH R01CA140554 (P.I. Yong J. Lee; David Bartlett; Herbert J. Zeh, III)
　　　　　　　　　P.I. 20%　　2010-2015　　Direct costs: $1,245,040　Indirect costs:$572,719
Title: Multimodality approach to hepatic colorectal metastases
The main goals of this proposal are to develop a novel strategy of isolated hepatic perfusion to treat unresectable liver metastases.

Pending

National Institutes of Health NIH R21 (P.I.: Yong J. Lee)
　　　　　　　　　P.I. 5%　2013-2015　$275,000
Title: Breast cancer stem cell heterogeneity and radiation sensitivity
The main goals of this proposal are to examine a correlation between breast cancer stem cell heterogeneity and prognosis. There is no scientific overlap.

National Institutes of Health NIH R21 (P.I.: Yong J. Lee)
　　　　　　　　　P.I. 5%　2013-2015　$275,000
Title: Strategy for optimizing breast cancer therapy
The main goals of this proposal are to develop a novel strategy for targeting breast cancer stem cells. There is no scientific overlap.

National Institutes of Health NIH R21 (P.I.: Yong J. Lee)
　　　　　　　　　P.I. 5%　2013-2015　$275,000
Title: Imaging and targeted-therapy for hepatic colorectal metastases
The main goals of this proposal are to develop a novel nanoparticle to treat hepatic colorectal metastase. There is no scientific overlap.

DOD-BC123009 (PI: Yong J. Lee)　Period: 6/1/2013-5/31/2016　5% effort
DOD Breast Cancer Program: Idea Award　　　　$375,000
Title: Application of multifunctionalized dendrimers in metastatic breast cancer imaging and therapy
The main goals of this proposal are to develop nanoparticles for imaging and therapy for breast cancer patients.

DOD-BC122984 (PI: Xinxin Song Mentor: Yong J. Lee)　Period: 6/1/2013-5/31/2016　DOD Breast Cancer Program: Postdoctoral Training Award　　　　$300,000
Title: Optimizing Molecular-Targeted Therapies in Breast Cancer
The main goals of this proposal are to develop a protocol treat breast cancer patients.

DOD Breast Cancer Program: Idea Award (PI: Juong G. Rhee, Co-investigator: Yong J. Lee)   Period: 6/1/2013-5/31/2016   5% effort $375,000
Title: Dormant Cancer Stem Cells in 3D multicell Spheroid and Therapy Resistance
The main goals of this proposal are to investigate the role of tumor microenvironment in radiosensitivity of breast cancer stem cells.

Previously Funded Grants

National Institutes of Health NIH RO1 GM074000 P.I.: Yong T. Kwon, Co-P.I. 3% 2006-2010, $1,856,976   "Proteomic of Ubiquitin-dependent N-end Rule Pathway"

National Institutes of Health NIH RO1CA48000   P.I. 50%   1988-1991 $180,000
   Mechanism of Drug-Induced Heat Protection

National Institutes of Health NIH RO1CA44550   Co-investigator, 10% 1989-1997 $1,346,682
   Preferential Killing of Melanized Tissues

National Institute of Health NIH RO1CA48000   P.I., 40%   1992-1997 $694,498
   Mechanism of Drug-Induced Heat Protection

National Institutes of Health NIH RO1CA53167 Co-investigator, 5% 1994-1997 $381,633
   Mild Temperature-Hyperthermia and LDR irradiation

National Institutes of Health NIH RO1CA48000   P.I., 40%   1998-2003 $972,932
    Stress Responses in Mammalian Cells

National Institutes of Health NIH RO1 CA95191   P.I. 30%   2003-2008 $1,322,540
    Metabolic Oxidative Stress and TRAIL Cytotoxicity

National Institutes of Health NIH RO1 CA96989   P.I. 30%   2004-2009 $1,893,750
"Role of Glutaredoxin in Metabolic Oxidative Stress"

National Institutes of Health RO3 P.I.: Yong J. Lee, 5%   2006-2008 $148, 500
                "Nutrients and Prostate Cancer Prevention"

National Institutes of Health NIH RO1 P.I.: Shivendra Singh, Co-P.I. 3% 2005-2008 $1,856,976
                "Prostate Cancer Prevention by Diallyl trisulfide"

The Susan G. Komen Breast Cancer Foundation P.I.: Yong J. Lee, 10% 2006-2009 $250,000
"Mechanisms of Breast Cancer-Preventive Effects of Organosulfur Compounds "

Elsa U. Pardee Foundation   P.I., 10%   1995-1996 $102,802
    Involvement of AP-1 Transcription Factors in the
                       Regulation of bFGF Gene

Elsa U. Pardee Foundation   P.I., 10%   1999-2000 $90,600
    Targeted Gene Thermotherapy

Elsa U. Pardee Foundation.   P.I. 10%   2002-2004 $116,491
              "HER-2/neu and Its Role in TRAIL-induced
     Cytotoxicity"

DOD-BC103217 (PI: Yong J. Lee)   Period: 3/1/2011-2/28/2012   5% effort
DOD Breast Cancer Program: Concept Award   Direct costs: $75,000 Indirect costs:$38,625
Title: Why are breast cancer stem cells resistant to radiation?
The main goals of this proposal are to investigate the role of Notch-Akt pathways in radiosensitivity in breast cancer stem cells.

DOD Prostate Cancer Program: DOD-PC001283   2001-2003 $98,000
     Mentor for postdoctoral traineeship
     Development of Gene Therapy with TRAIL for Prostate Cancer

DOD Prostate Cancer Program:DOD-PC010270   2002-2004 $98,000
Mentor for postdoctoral traineeship
"TRAIL-based Radio-Gene Therapy for
Prostate Cancer"

DOD Prostate Cancer Program:DOD-PC040833   2004-2006 $125,000
Mentor for postdoctoral traineeship
"Modulation of TRAIL Cytotoxicity by Amiloride in Prostate Cancer"

DOD Prostate Cancer Program: Idea development Award: DOD-PC020530
                         P.I.   10%   2003-2007 $503,575
              "Effect of HER-2/neu Signaling on Sensitivity to TRAIL in Prostate Cancer"

Competitive Medical Research Fund.   P.I., 10%   2002-2003 $25,000
              "Stress Responses in Mammalian Cells"

        Pittsburgh Foundation   P.I., 5%   2003-2005 $125,000
"Role of HER-2/neu in TRAIL-based Therapy", $125,000

William Beaumont Hospital Research Institute (WBHRI) 88-15
                         P.I., 15%   1988-1989 $74,812
              Mechanism of Heat-Shock Protection

WBHRI 89-02   P.I., 15%   1989-1990 $24,000
              Mechanism of Heat-Shock Protection

WBHRI 90-06   P.I., 15%   1990-1991 $66,928
     Characterization and Purification of 26 kDa Protein

WBHRI 91-10   P.I., 15%   1991-1992 $71,338
     Mechanism of Super-thermotolerance Development

WBHRI 92-28   P.I., 15%   1992-1993 $72,698
     Protein Synthesis-Independent Thermal Resistance

WBHRI 92-27   P.I., 15%   1992-1993 $76,573

Physiological and Biochemical Functions of HSP70

WBHRI 93-09   P.I., 15%   1993-1994 $42,931
    Physiological and Biochemical Functions of HSP70

WBHRI 93-21R   Sponsor   1993-1994 $7,000
    Interferon and Hyperthermia:Effects on Adriamycin
        Cytotoxicity in Human Carcinoma Cell Lines

WBHRI 94-14   P.I., 15%   1994-1995 $73,275
    Physiological and Biochemical Functions of HSP70

WBHRI 94-47M   P.I., 5%   1995-1996 $5,000
    Proto-oncogene and Breast Cancer Angiogenesis

WBHRI 95-07   P.I., 15%   1995-1996 $57,744
    The Role of PKC in the Regualtion of Heat Shock Gene Transcription

WBHRI 95-38RM   Sponsor   1995-1996 $5,000
    The Role of Protein Kinase C in the Regulation of
    Basic Fibroblast Growth Factor Gene

WBHRI 96-03 AP-1   P.I., 15%   1996-1997 $67,164
    Transcription Factors and bFGF Gene Expression

WBHRI 97-06   P.I., 10%   1997-1998 $84,480
    Application of Stress-Inducible Promoters in
    Targeted Gene Therapy

WBHRI 97-22M   Sponsor   1997-1998 $5,000
    Retroviral Transfer of Nitric Oxide Synthase into
    Breast Carcinoma Cell

Oral Cancer Center Pilot Project Program at the University of Pittsburgh
NIH PO1   P.I., 10%   2002-2003 $29,900
"Role of HER-2/neu in TRAIL-induced
Cytotoxicity in Oral Cancer"

Society Activities:

1997 North American Hyperthermia Society Program Committee
2001 NASH refresher course introducer
2001 President for Korean Scientists Meeting in Pittsburgh

Study Section

2001   The Susan G. Komen Breast Cancer Foundation (FY 2001 Tumor Cell II Review Committee)
2002   NIH AdHoc Reviewer (Radiation Study Section)
2003   The Susan G. Komen Breast Cancer Foundation (FY 2003 Tumor Cell I Review Committee)

2004	The Susan G. Komen Breast Cancer Foundation (FY 2004 Tumor Cell I Review Committee)
2005	The Susan G. Komen Breast Cancer Foundation (FY 2005Tumor Cell I Review Committee)
2005	NIH AdHoc Reviewer (Cellular Signaling and Dynamics Study Section)
2006	The Susan G. Komen Breast Cancer Foundation (FY 2006 Tumor Cell I Review Committee)
2007	CCSG Review Committee

Editorial Board

Clinical Medicine Insights: Urology
Korean Journal of Biological Science
PLoS One